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XXXIX. THE CHEMICAL NATURE OF A BACTERIAL HAEMOLYSIN.

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(Received July 17th, 1914.)

I. THE RELATION BETWEEN THE VELOCITY OF HEAT-DESTRUCTION OF A BACTERIAL HAEMOLYSIN AND THE TRUE REACTION OF THE SUBSTRATE.

Our present knowledge of the effect of heat, and also of acid and alkali on the velocity of destruction of bacterial haemolysins, is due to Famulener and Madsen [1908]. They were able to show that in all cases, whether by altering the temperature or the degree of acidity or alkalinity of the medium, the process of destruction proceeded as a reaction of the first order—that is to say, the destruction at any instant was proportional to the amount of haemolysin then present. They found that as they increased the quantity of acid or alkali, the velocity constant increased and there was a minimum velocity somewhere in the neighbourhood of the neutral point. The exact relationship of this minimum to neutral reaction of the medium, they did not determine however, as the hydrogen ion concentration was not measured. Since that time the technique of the determination of the hydrogen ion concentration of biological liquids by the electrical method has greatly developed and at the present day we can find its value with a very considerable degree of accuracy. It is therefore interesting to investigate how the velocity of destruction varies with the hydrogen and hydroxyl ion concentration. The fact that the velocity increases both with acidity and alkalinity is strongly in favour of the view that the substance producing haemolysis is an amphoteric electrolyte and that both anion and cation are thermolabile, while the undissociated molecules are probably not at all so, under the conditions of the experiment. On this assumption the velocity should be at a minimum when the anions and cations are present in the fewest possible numbers, that is at the isoelectric point. From such experiments therefore

we ought to obtain information about the relative magnitudes of the acidic and basic dissociation constants of the haemolysin. We shall then know whether the haemolysin functions as a weak acid or base in pure watery solution.

The haemolysin I have used is that produced in broth cultures of *Vibrio nasik*. After growing this micro-organism in alkaline broth (such as is employed for the production of diphtheria toxin) for a period of nine or ten days at 37°, it is killed by toluene and the dead bacteria removed by a centrifuge at a speed of about 5000 revolutions per minute. The clear liquid is separated and toluene added. The haemolysin keeps well at room temperature. It is lytic for the red corpuscles of many different animals. I have used a 1 % suspension of sheep red cells in 0.9 % NaCl. The relative amounts of haemolysin present in a series of samples were estimated by Madsen's colorimetric method [Kraus and Levaditi, 1908].

The reaction of nasik lysin was altered by the addition of HCl or NaOH according as an acid or alkaline medium was required. Small amounts of acids simply had the effect of neutralising or lessening its degree of alkalinity—the lysin being alkaline to begin with. In each case a definite amount of acid or alkali was added to 90 cc. of lysin and the whole made up to 100 cc. with distilled water. It was then left overnight in the cold room and next day hydrogen was bubbled through for three hours. This procedure dissipated the traces of toluene which were still dissolved in the lysin, and which Sørensen has shown may adversely affect the electrodes in the electrical method of estimating hydrogen ion concentration. It also had the effect of removing any excess of CO₂ or NH₃. Carbon dioxide is not an electrically active gas, that is to say, it does not give rise to an electromotive force in a platinum electrode dipping in a liquid, but when dissolved in water it forms H₂CO₃ which is slightly dissociated into H⁺ and HCO₃' ions and therefore increases the acidity of the solution. This would not matter except for the fact that the CO₂ easily diffuses out of the solution, notably into the atmosphere of hydrogen during electrical measurement of the reaction, thereby altering the reaction of the liquid and the saturation of the electrode. Ammonia on the other hand is electrically active and would modify the potential of the hydrogen electrode. In the most alkaline samples of lysin with which I worked there was a very distinct smell of NH₃, due no doubt to decomposition of ammonium salts by the added NaOH and so it was very necessary to get rid of this. The lysin was therefore left in contact with hydrogen for another day in the cold room. The most suitable temperature at which the destruction of the lysin could be followed, was shown by

Famulener and Madsen's experiments to be about 46.0° and this temperature was therefore chosen. Immediately before the heating of the lysin took place, its hydrogen ion concentration $[H^+]$ ¹ was measured by the electrical method. The particular type of apparatus used was that described by Michaelis and Rona [1909] the characteristics of which are the *still* atmosphere of hydrogen combined with *minimum immersion* of the electrode. When the electrodes only just dip into the liquid the E.M.F. becomes constant very quickly and this is often of great importance in biological work where the reaction may be altering during the operation. The measurements took place at 46.0 — the same temperature as that at which the destruction of lysin was followed. As normal electrode I used a hydrogen electrode dipping into $N/10$ HCl. The $[H^+]$ of this solution at 46° is only slightly less than at ordinary room temperature and it was estimated by graphic interpolation from data given by A. A. Noyes and others [1910] to be dissociated to the extent of 91.4 %. As connecting liquid KCl was used and readings taken with two strengths, namely, 1.75 N and 3.5 N solutions and the true value extrapolated by addition of the difference of the two potentials to the latter according to the method suggested by Bjerrum [1905]. About two minutes was allowed for the cells to attain the temperature of the water bath and then readings taken immediately. Potential equilibrium is practically established by this time as one would expect, especially as the hydrogen electrodes have been in contact with the liquids to be tested for some minutes previous to immersion in the water bath. By continuous observation of the E.M.F. at 46° a very gradual change is perceived due to slight alterations of the reaction of the lysin, as will be mentioned later. An immediate reading as soon as the lysin has reached 46° is necessary therefore and although an error may creep in here it can only be trifling.

The reaction of a given sample of lysin being ascertained, it was next heated to 46.0° . For this purpose a water bath with a toluene regulator was employed. During the heating the temperature never varied more than ± 0.025 and often was practically constant, the thermometer used being an accurate one graduated in tenths of a degree and read with the naked eye. Arrhenius has introduced an empirical formula for the relation between velocity constants and temperature, namely,

$$\frac{k_1}{k_2} = e^{\frac{\mu}{2} \left(\frac{T_2}{T_1} - \frac{T_2}{T_2} \right)}$$

¹ H^+ stands for hydrogen ion, the brackets for concentration, and p_{H^+} for the negative exponent when the normality is expressed as a power of 10.

k_1 and k_2 being the velocity constants at the absolute temperatures T_1 and T_2 and μ a constant for a given substance. Famulener and Madsen [1908] have shown that this equation is valid for nasik haemolysin, where $\mu = 128,000$. We can therefore calculate what would be the change in the velocity constant for a rise in temperature from 45.975° to 46.025° . Under these conditions the ratio $\frac{k_1}{k_2} = 1.032$, which represents the maximum error and is so small as to be negligible. The lysin was placed in a large test tube containing the thermometer, which was also used for stirring, and fitted with a large cotton wool plug. It was previously heated to about the required temperature and quickly transferred to the water bath. As soon as the thermometer registered exactly 46.0° a sample was removed by insinuating a narrow bore glass tube at the side of the wool plug, the top of which was fitted to a 10 cc. pipette with a piece of rubber tube. From the pipette 7 cc. were measured off into a tube containing sufficient acid or alkali to neutralise the added alkali or acid, and immediately cooled down in ice-cold water. By this manœuvre the temperature of the lysin in the water bath did not appreciably alter. The attempt to bring the reaction back to its original value by adding the calculated amount of acid or alkali as the case might be, was only partially successful as can be easily imagined, but this is not of great importance since the haemolytic action of the lysin on red cells, within fairly wide limits, is independent of the $[H']$. After seven samples had been removed at definite intervals of time the $[H']$ of the remainder was determined as before. It was found that in practically every case the reaction had changed during heating to a greater or less extent. It might be thought that it would have been better to alter the reaction by means of regulator mixtures which keep the reaction constant, that is, weak acids or bases in conjunction with their salts, instead of HCl and NaOH. There are however objections to this. In the first place I was desirous of introducing, if possible, only ions of the kind already present. Evidence is accumulating that other anions and cations besides H ions and OH ions, influence the chemical actions of biological substances and so may have altered the chemical relation of the lysin to H and OH ions. In addition, the amount of regulator necessary would be relatively large, and the dilution of the lysin would be such as materially to limit the range over which the destruction could be studied. As can be seen from Fig. 1 the alteration of reaction during heating is in most instances relatively slight and in any case insufficient to vitiate the main results of this investigation. Fortunately the greatest changes took place

in the neighbourhood of the neutral point, where considerable alterations in the reaction produce relatively small changes in the velocity constant.

The following will serve as a type of a single determination of a velocity constant.

1 N.HCl	H ₂ O	Nasik lysin	[H ⁺]
2.2 cc.	7.8 cc.	90 cc.	p _H at 46.0°
			5.47

Heated at 46.0°.

Minutes	Haemolytic Equivalents, Volumes of lysin producing equal haemolysis	$\left(k = \frac{1}{t_2 - t_1} \log_e \frac{A - x_1}{A - x_2} \right)$ $k \times 0.4343$
0	0.055	
15	0.065	0.00484
30	0.07	0.00214
45	0.072	0.00082
60	0.075	0.00118
75	0.09	0.00528
90	0.11	0.00581
	Mean	0.00335

To 7 cc. of each sample after heating were added 0.154 cc. of N.NaOH and 0.846 cc. of water. After heating at 46°, p_H was found to be 5.51.

TABLE I. *Velocity of Destruction of Nasik Lysin at Different Reactions.*

$\left(k = \frac{1}{t_2 - t_1} \log_e \frac{A - x_1}{A - x_2} \right)$ $k \times 43.43$	p _H at 46.0°	
	before heating	after heating
28.0	8.43	8.49
18.93	8.30	8.23
15.7	8.01	8.17
9.84	8.19	8.10
5.2	7.55	7.62
2.95	7.37	7.17
0.72	7.06	6.95
0.4	7.06	6.62
0.08	6.54	—
0.22	6.46	6.18
0.16	5.82	5.49
0.335	5.48	5.5
2.05	5.17	5.07
3.95	5.04	5.04
6.4	5.10	4.88
10.6	5.00	4.82
16.95	4.77	—

In this way a number of values for k were obtained between two limits of [H⁺] in each case. The results have been tabulated and charted. An inspection of Fig. 1 will reveal the points of interest.

The lines through which the curve has been drawn represent the limits of the change of reaction during heating. The velocity of destruction rises

with increasing acidity and also with increasing alkalinity though not so abruptly in the latter case. The minimum does not correspond with the neutral point but is slightly on the acid side.

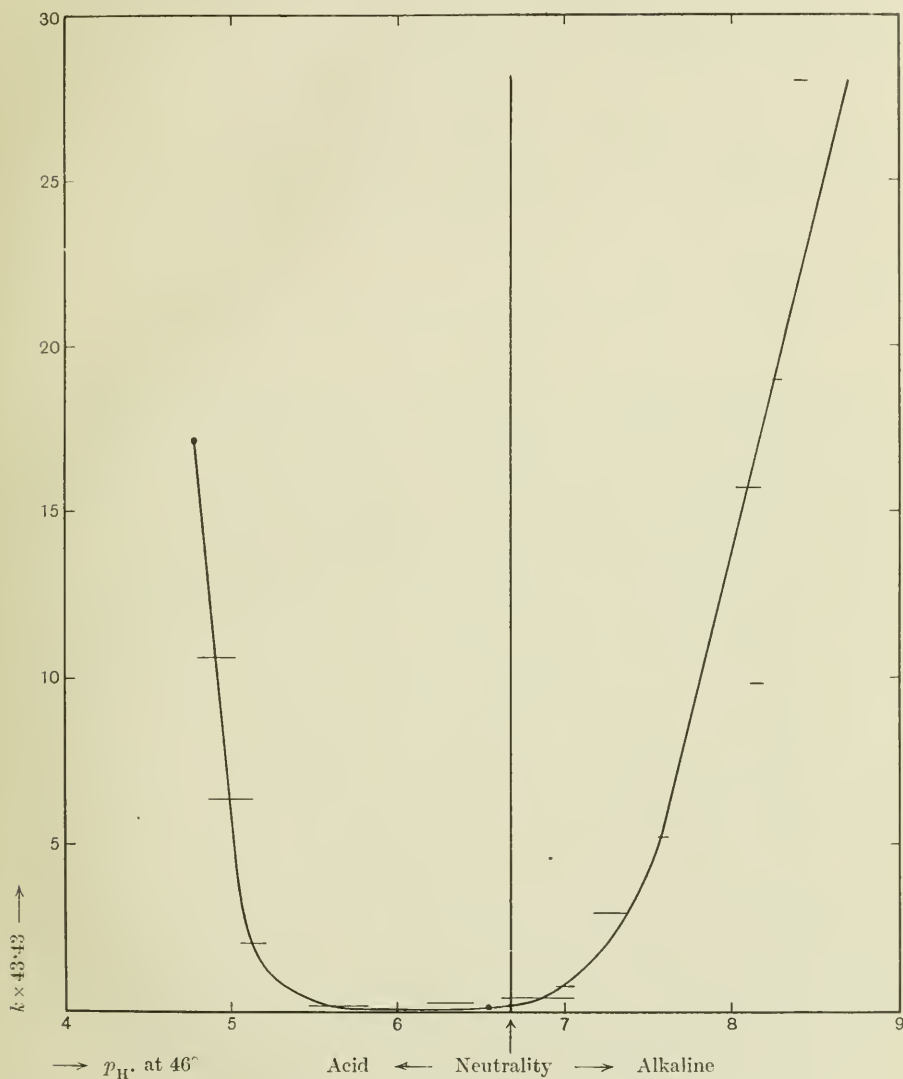


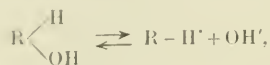
Fig. 1. Relation between Velocity of Destruction of Nasik Haemolysin and the Reaction of the Substrate at 46.0°.

INTERPRETATION OF RESULTS.

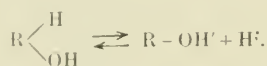
These facts can best be explained on the assumption that the substance causing haemolysis is an amphoteric electrolyte with an acid dissociation constant greater than the basic, that is $k_a > k_b$. If we represent an ampholyte (amphoteric electrolyte) in the usual way



then in strongly acid solution it will be ionised practically entirely thus:



and in strongly alkaline solution



Both the anion and cation are thermolabile and we should expect that the velocity of destruction would be at a minimum at the isoelectric point (I.P.) because here the total number of anions and cations is a minimum. The I.P. is by definition the $[\text{H}']$ of the medium when the anions and cations of the ampholyte are present in equal numbers and it can also be shown that their total concentration is the least possible at a given temperature and in a given solvent—in other words at the I.P. the molecules of an ampholyte suffer least dissociation in solution. It is easy to see that if the H' and OH' have the same tendency to split off from the molecule ($k_a = k_b$) the I.P. will correspond exactly with the neutral point; but if, for example, there is a greater tendency for the H' than the OH' to dissociate, as is the case with so many protein substances, it will be necessary, in accordance with the law of mass action, to add an acid to limit the splitting off of H ions in order that an equal number of anions and cations may be present. Now the neutral point at 46.0 obtained by graphic interpolation from data for the dissociation product of water at different temperatures given by Lundén [1908] is $p_{\text{H}}, 6.69$. On reference to Fig. 1 the minimum of the curve appears well on the acid side of the neutral point, and the explanation is that the lysin has a larger acid than basic dissociation constant. These results are consistent with the view that the lysin is an ampholyte comparable with the serum proteins.

There is another possibility, however, namely that the lysin is a dibasic acid. A minimum velocity of destruction would then correspond with a

maximum number of monovalent anions present, and the velocity would increase on the one hand with the number of undissociated molecules of lysin, and on the other hand with the divalent anions, according as the solution was made more acid or less acid respectively.

Trypsin is an ampholyte in which only the monovalent anion has a ferment action, but it seems probable that divalent anions which are inactive are formed by splitting off of another H^+ on further reducing the acidity [Michaelis and Davidsohn, 1911]. Similar considerations would apply in the case of a di-acid base. It is only possible to choose between the two alternatives by testing the direction of movement in an electric field, in solutions of varying $[H^+]$.

II. CATAPHORESIS EXPERIMENTS WITH NASIK LYSIN.

The lysin already employed was not suitable for this purpose on account of its high electrical conductivity. The original broth on which the vibrio was cultivated contained 0.5 % NaCl in addition to salts from the meat and peptone. I first attempted to produce a lysin in a salt-poor medium—peptone water alone or with a little broth added—but I only succeeded in obtaining a weak lysin in this way. Dialysis was next tried. Parchment paper proved useless as the lysin disappeared almost as quickly as the salts from the solution. Much better results followed the use of an animal membrane as dialyser (the so-called Fischblase), which has been successfully used to obtain electrolyte-free ferment solutions. By dialysing for 24 hours against distilled water, I was able to get a fairly strong lysin with an electrical conductivity about quarter that of N/100 NaCl. Reduction of lytic power occurred here also, but to a much less extent than in the case of the parchment membrane.

The apparatus used was that designed by Landsteiner and Pauli, with unpolarisable electrodes of Ag in NaCl, and Cu in $CuCl_2$ which have been recommended by Michaelis. In order to vary the $[H^+]$ a regulator mixture was used as it did not seem probable that the reaction would remain sufficiently constant during the experiment by the simple addition of HCl. It was not necessary to make the solution more alkaline than it already was, as the lysin migrated to the positive pole without any addition.

The most suitable mixture to produce the required range of $[H^+]$ was lactic acid and its sodium salt. This regulator has been used several times in the case of ferments and the lactate ion has not been found to have

any specific effect. This regulator was added to the lysin in the ratio of regulator : lysin = 1 : 9. It is not practicable to dilute the lysin more as its strength would be insufficient to make it easy to detect when it had moved to one or other pole in small quantity. Its content of added salt (sodium lactate) was 0.02 N. This is not very concentrated for a regulator but it seemed desirable to keep the electrolyte content as low as possible. The slaking power of the lysin on the $[H^+]$ of the regulator was considerable. After several mixtures had been made and the values of their $[H^+]$ estimated it was easy to construct a curve from which to predict fairly accurately in advance the $[H^+]$ of any mixture. Such mixtures were put in the middle compartment of the cataphoresis apparatus, and in the side pieces a regulator mixture of as nearly as possible the same $[H^+]$, with the lysin replaced by distilled water. The latter mixture was therefore of slightly lower specific gravity than the lysin mixture, but had the same amount of added electrolyte, if we neglect the slightly dissociated anions of the lactic acid. The electrodes were filled with 0.01 N NaCl in addition to the small amount of concentrated salt solutions—NaCl on the positive side, and $CuCl_2$ on the negative side—which formed layers at the bottom. The whole apparatus when filled was air-tight, and was sunk in a water bath at 10° . A pressure of 200 volts was then applied for four hours. The current passing was of the order of 0.25 milliampere, but varied of course with different conditions of the solutions, the concentration of electrolyte at the electrodes and the size of the electrodes. At the end of the time allowed, 0.4 cc. of liquid was removed from the bottom of each side tube of the apparatus with a Jena glass pipette, which was drawn out to a very fine capillary and was fitted to a syringe by means of a small rubber cork. Very evenly regulated slow suction was produced in this way, so that the bottom layer of liquid just above the taps in the side tubes could be abstracted without mixing with the supernatant layers. To this liquid was added 0.2 cc. of a 3.6 % solution of NaCl, to bring its tonicity up to the level which the red blood cells can tolerate without becoming haemolysed. It was then carefully neutralised with 0.2 N NaOH. The technique of neutralisation presented some difficulties at first owing to the fact that it was not feasible to waste more than a minute amount of liquid in the process, and it was very necessary to bring the solution near neutrality or else haemolysis would be produced by the excess of acid or alkali present. Very small drops were taken and tested with equally small drops of suitable indicators such as methyl-red, rosolic acid, α -naphthol-phthalein, on a white porcelain plate. It was found

absolutely essential to use Jena glass for the pipettes for removal of the liquid and indicators. In this way it was easy to neutralise with a very small total loss of liquid. Lastly 1.0 cc. of a 1 % suspension of sheep red cells in 0.9 % NaCl was added, and the whole incubated for three hours at 37°. The tubes were then shaken and examined next day for haemolysis. A similar sample was always taken from the middle compartment at the end of cataphoresis to show that the lysin had not been destroyed by excessive acidity or other circumstances (see later). In all the mixtures of lysin and regulator a turbidity appeared immediately which gave rise to more or less precipitate as time went on, according to the degree of acidity.

This precipitate is presumably mainly composed of denaturated protein coming from the meat used in the preparation of the original broth, which only completely flocculates when brought to its isoelectric point. At any rate a similar precipitate can be obtained from a meat broth (without peptone) with its optimum at about the same $[H^+]$. The presence of this protein impurity in the lysin solution was a considerable complication. At certain degrees of acidity it had the power of removing much of the lysin from the solution, in the process of precipitation, leaving only a weak lysin to work with. Before discussing its effect on the direction of migration of the lysin I will indicate the experimental results.

Table II shows the direction of movement of the lysin in an electric field at varying $[H^+]$. The temperature was 10° and the $[H^+]$ was determined at the same temperature using in this case a calomel electrode as standard. The π_0 value (0.3383) at 10° was obtained by graphic extrapolation from values given by Sørensen [1912]. It will be seen that in most cases there is not very much difference between the $[H^+]$ of the lysin before and after cataphoresis. The liquid on the + side in every case became more alkaline; that on the - side sometimes increased in acidity but showed less tendency to change from the original reaction of the regulator. It is obvious that these small deviations from the reaction of the lysin in the middle compartment will not militate against the definition of the limit of movement to either pole. For instance a lysin anion travelling towards the + pole on coming in contact with a more alkaline medium will not tend to have its charge reversed as it would if it met a more acid medium, and it will proceed on its way. Similarly on the acid side increased acidity will not interfere with the progress of the lysin cations. The charge and therefore the direction of movement of the ions will thus be determined by the $[H^+]$ of the lysin. It will be seen that movement to both poles has been obtained

TABLE II. *Cataphoresis of Nasik Lysin*

Temperature 10° Duration, 4 hours			E.M.F. 200 volts.			k_w at 10°, 10°			
			P_H						
			Before cataphoresis			After cataphoresis			
Lysin, No.	So Lactate, cc.	0.1 N. Lactic acid, cc.	H ₂ O cc.	Lysin Regulator (middle compt.)	Lysin		Movement Pole	Remarks	
					Regulator	(middle compt.)			
1	9	0.5	0.425	3.92	4.00	3.87	4.23	3.88	+
2	"	"	0.4	3.77	3.94	3.85	4.28	3.92	+
3	"	"	0.15	3.60	3.60	3.59	3.85	3.63	+
4	"	"	0.2	3.29	3.26	3.23	3.55	3.25	0
5	"	"	0.4	3.01	2.87	3.01	3.02	2.9	0
6	"	"	0.5	2.94	2.99	2.92	3.10	2.97	-
7	"	"	0.5	2.94	2.70	—	2.9	2.78	-
8	"	"	0.7	2.75	2.73	2.82	3.02	2.74	?

All lysin destroyed at end of exp., due to extreme acidity.

No movement of visible particles. Strength of lysin after exp. much reduced.

- liquid turbid.

No movement of visible particles. Strength of lysin after exp. much reduced.

+ liquid turbid.

- liquid turbid.

All lysin destroyed at end of exp., due to extreme acidity.

with a zone of no movement between them, according to the degree of acidity. This points to the fact that the lysin is an ampholyte and lends additional support to the interpretation of the experiments on velocity of destruction of the lysin by heat.

DISCUSSION.

The early experiments to determine the electric charge of antigens (toxins, etc.) were all vitiated by neglect to exclude sharp changes of reaction at the electrodes. Field and Teague [1907] used platinum electrodes and in addition complicated their work by the presence of agar, through which they designed the toxin should travel and lend itself to easy detection. They determined that both the toxin and antitoxin of diphtheria and tetanus had positive charges which were not reversed by altering the reaction of the solvent—a most unlikely result in the light of later research. Teague and Buxton [1907] believed that they had shown that haemagglutinins moved to the cathode and considered that “all the active principles of serum concerned in the anti-reactions have a tendency to migrate towards the cathode.” Their experiments were carried out in a similar way to those of Field and Teague except that they used unpolarisable electrodes. Bechhold [1907] used membranes with the idea of excluding the products formed at the poles from the substance being examined.

Landsteiner and Pauli [1908] in the case of abrin, ricin, and the haemagglutinin found in normal fowl serum, obtained a movement to the negative pole in each. A determination of the $[H^+]$ of the solution was here lacking, but it was presumably not far from the neutral point. These experiments are an advance on the previous ones as the fallacies of the method were eliminated.

Landsteiner [1913] states that he has some unpublished results showing that staphylolysin and arachnolysin are amphoteric. The only details given are that in weak acid reaction the lysins migrated to the negative pole, and in weak alkaline to the positive pole.

Let us now attempt to co-ordinate the two series of results, namely, the curve of velocity of destruction and the movement in an electric field. The temperature in the two cases was different because the minimum velocity of destruction of the lysin had to be reduced for the cataphoresis experiments, so that the lysin should not be appreciably weakened by the end of the time allowed. Suppose for the present we estimate the I.P. at 10° (Table II) at about $p_H \cdot 3.3$. At 46° the minimum velocity of destruction

can be read from the curve at roughly p_H 6.1. Is it reasonable to suppose such a difference in the I.P. would occur at the two temperatures? In the first place it must not be forgotten that values of $[H']$ for different temperatures are not directly comparable. For instance at 46° the $[H']$ of neutral reaction is p_H 6.69, but at 10° the $[H']$ of neutral reaction is p_H 7.26. It is obvious that these two values are thermodynamically equivalent. It is in fact the ratios H'/OH' , which correspond at two different temperatures, this ratio being of course equal to unity at neutrality. A simple calculation shows that the ratios of H' to OH' will be the same at the two temperatures when the p_H is 6.1 at 46° and 6.67 at 10° . In other words there is a difference of I.P. corresponding to the difference between p_H 6.67 and p_H 3.3 to be accounted for. We have now to see what is the effect of temperature on the other amino-acids. The only data I have been able to find in the literature are those of Lundén [1906]. He determined the acid and basic dissociation constants of three simple amino-acids at 25° and 40° . I have calculated the shift of I.P. in each case, by means of the equation $R = \frac{I^2}{k_w}$

[Michaelis and Rona, 1910].

Ampholyte	Temp- erature	k_a	k_b	$\frac{k_a}{k_b} = R$	Iso-electric Point	
					$R = \frac{I^2}{k_w}$ p_H	at $25^\circ = 10^{-14}$ at $40^\circ = 10^{-13.19}$
Acetoxime	25	6.0×10^{-5}	6.5×10^{-13}	0.92	7.02	
	40	9.9×10^{-5}	19.0×10^{-13}	0.52	6.89	
β -Asparagine	25	1.35×10^{-9}	1.53×10^{-12}	882.4	5.53	
	40	3.22×10^{-9}	4.23×10^{-12}	761.2	5.56	
<i>o</i> -Amino benzoic acid	25	1.06×10^{-4}	1.38×10^{-12}	7.68×10^6	3.56	
	40	1.35×10^{-4}	3.16×10^{-12}	4.37×10^6	3.68	

The basic constant in each case increases more than the acidic with rise of temperature, which leads to a displacement of the I.P. towards the alkaline side. In the case of acetoxime the basic constant is greater than the acidic and the I.P. is consequently on the alkaline side of neutrality, but in the last two the I.P. is on the acid side. Allowing again for the fact that the values of I.P. need correction as they are determined at different temperatures, which correction as we have already seen still further increases the difference, we find that the nett result of a rise of temperature on the I.P. in the case of ampholytes like β -asparagine and *o*-amino-benzoic acid, is to shift it towards the neutral point. This is exactly what has taken place with the lysin. It is true that the difference in the last case is much greater than

the more extended range of temperature would account for and it is quite possible that there are other factors concerned. It must be remembered, for instance, that the salt content of the lysin solution in the heat destruction experiments was very different from that in the cataphoresis experiments. However the lysin is in all probability much more complicated than such simple ampholytes as the ones cited, so that it is unwise to expect too close a parallel. From this point of view therefore the experimental facts are in harmony with the theory that the lysin is an ampholyte.

We have still to consider the effect of the presence of other substances on the behaviour of the lysin. I have already drawn attention to the fact that it is not pure. In biochemistry we are constantly faced with the difficulty that the body we are investigating is mixed with other substances which may influence its action. There is some experimental evidence bearing on this point. Firstly there are some anomalous instances of heat destruction of haemolysins. Madsen, Famulener, and Walbum [1904] found that the haemolysin of *staphylococcus* was greatly reduced in strength at 70° , but almost completely returned on raising the temperature to 100° for five minutes. Landsteiner and Rauchenbichler [1909] found the same thing and attributed it to a union of staphylo-lysin at a medium temperature with some other substance in the solution, which compound was dissociated again at 100° . Working with megatheriolysin I was able to show [Atkin, 1910] that the velocity of destruction increased up to a temperature of about 55° , then it decreased up to a temperature of 75° , after which it increased up to 100° . This phenomenon is probably due to the same cause as that operating in the case of staphylo-lysin. I also repeated Madsen and Famulener's experiments without being able to confirm their results—the staphylo-lysin showed only a gradually increasing destruction with rise of temperature. This seems further to substantiate the view that the return of activity at a high temperature is not a characteristic of the lysin itself, but due to the influence of some foreign substance. Naturally in different samples the quantitative if not the qualitative relations of the several constituents will vary. No such property exists in the case of nasik haemolysin however, possibly because of its relatively great thermolability, for it is completely destroyed at a temperature considerably less than that at which the supposed union takes place in the case of staphylo-lysin and megatheriolysin. Similar considerations also apply to tetanolyisin. If the hypothesis that megatheriolysin undergoes a union with some other body at certain temperatures, which modifies its destruction by heat, is correct, the process of destruction

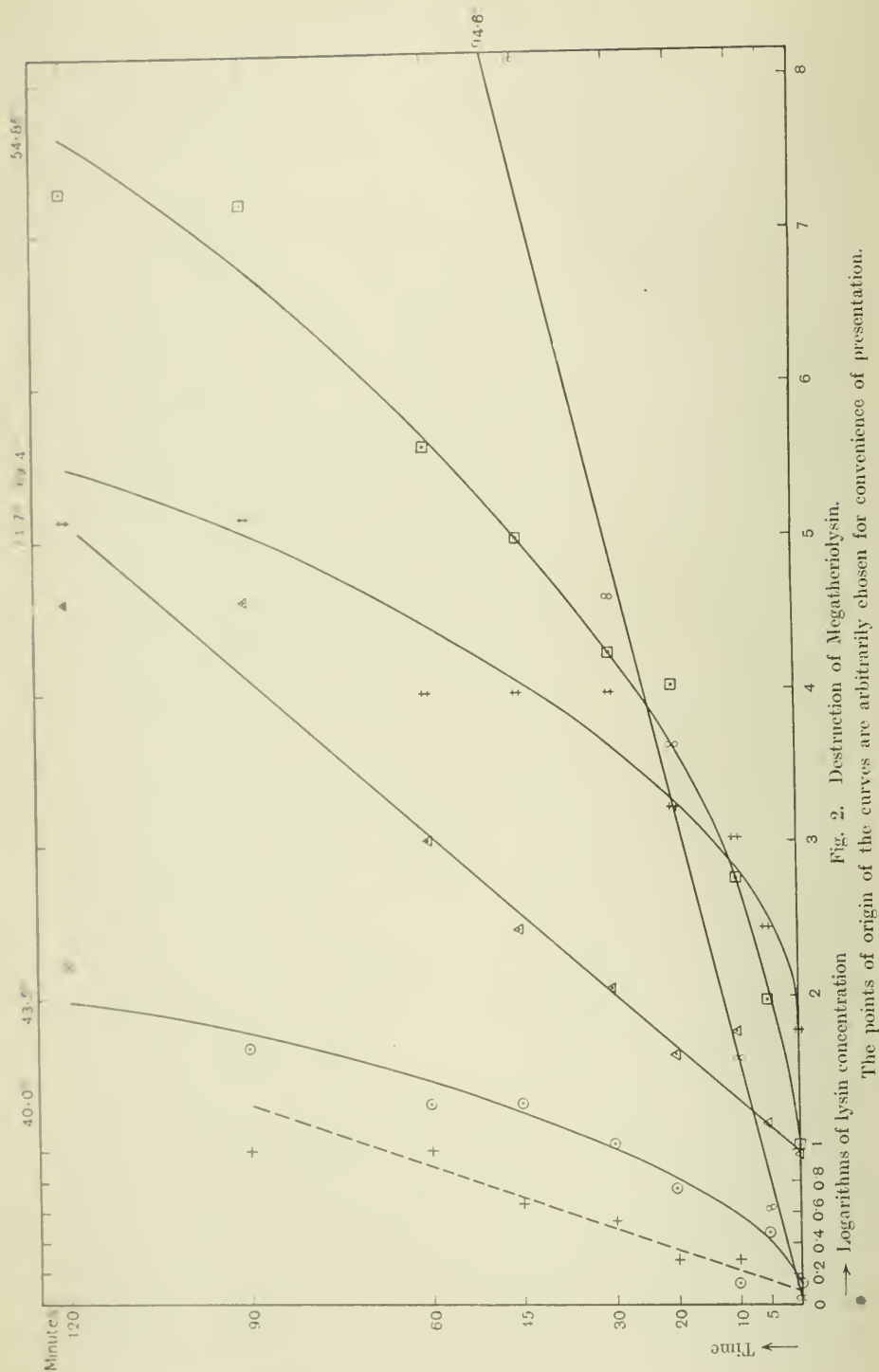


Fig. 2. Destruction of Megatheriolysin.

→ Logarithms of lysin concentration

The points of origin of the curves are arbitrarily chosen for convenience of presentation.

should not be a unimolecular one. In other words, if we plot the logarithms of lysin concentration against time we ought not to get a straight line. If, on the other hand, at higher or lower temperatures such a combination either does not take place or is disrupted, it is reasonable to expect the process to be then a logarithmic one. I have applied this test to my experimental results in the case of megatheriolysin [Atkin, 1910] and find it actually to be so.

As the curves show (Fig. 2) the process of destruction is only unimolecular, firstly from a temperature of about 74° , upwards, which is near the point at which the rate of destruction again begins to increase with temperature; secondly, below a temperature of about 43° . It is not quite certain that the destruction at 40.0° is logarithmic, but at any rate it approximates closely to a straight line, if it is in reality a curve. It has been drawn in the figure as a dotted line. This last point is interesting as it shows that the influence of the foreign substance is being felt at a temperature considerably lower than that at which it is able to decrease the velocity of destruction with rising temperature. It will be noticed that the hypothetical union with another substance is not a reversible process, otherwise the decomposition which presumably takes place at high temperatures would result in a re-union on cooling down, for we must remember that the actual test on red blood cells is carried out at 37° . In fact the observation that we are able to detect the phenomenon at all renders it highly probable that it is irreversible, for if this were not so we should expect the union at medium temperatures to be split up again on cooling to 37° . We seem to have a criterion therefore with regard to the abnormal destruction of lysins by heat, namely, that if at any temperature it can be shown that the destruction is *not* unimolecular, the velocity of destruction will not rise regularly with temperature, or what is another phase of the same phenomenon, the strength of the lysin will decrease at a certain temperature, and reappear again at a higher one.

In my present experiments with nasik lysin the temperature was kept constant at 46° , the reaction alone being changed. It is hardly to be expected that a union with another body would take place having the same effect as in the case of megatheriolysin, seeing that this effect requires a considerably higher temperature, but nevertheless I have subjected the figures relating to the velocity of destruction to the same test and find that in every case the process is unimolecular. This fact is in favour of the chemical freedom of nasik lysin, under the conditions of these experiments.

Work bearing on the influence of impurities on the movements of enzymes in an electric field has been carried out by Pekelharing and Ringer [1911]. One of them devised a method of purifying crude pepsin by dialysing and thus reducing the acidity, so that the enzyme partially separates out. They do not claim that their product is absolutely pure, but at any rate it is more nearly pure than any commercial preparation. With it they obtained migration to the anode only, within the limits of acidity they tried. Addition of caseinogen, crystallised serum albumin, or albumose (Witte's peptone), caused migration more or less to the cathode. The pepsin was in fact carried by the albumin, etc. They were of opinion that the value 5.5×10^{-3} found by Michaelis and Davidssohn [1910] for the i.p. of pepsin was not reliable.

In the present case the lysin is mixed with, at any rate, one other protein substance which flocks out at a definite $[H]$. It is difficult to say whether the lysin also flocks out at its i.p. as its absolute concentration must be extremely small and at 10° the i.p. apparently nearly coincides with that of the protein impurity. A glance at Table II, No. 4, shows that at this point there is no movement of the visible particles and at the same time the solution has become depleted of most of its lysin. We may here have a condition of things where the lysin and visible precipitate have opposite electric charges and consequently a union has taken place. The lysin would then presumably move with the precipitate. If this were so this boundary of the i.p. or rather isoelectric zone of the lysin would be rendered obscure. When however the sign of the charge of the lysin changed, the two bodies would again have similar charges and the lysin would be free to move independently. Similar considerations apply to the other boundary because the visible particles may become negatively charged before the lysin. A rough approximation to an isoelectric zone is therefore all that can be attained. If we take this zone as p_H 3.0 to 3.6 and assume that the geometric mean represents the i.p. then this will be the arithmetic mean of the p_H values, namely p_H 3.3. Therefore approximately,

Isoelectric point at 10°	p_H 3.3.
16°	p_H 6.1.

This represents a large decrease in acid character with rise of temperature. It is interesting to attempt a determination of the acid and basic dissociation constants. Michaelis and Menten [1913] have devised a graphic method of obtaining these constants. Using the velocity constants I have constructed

dissociation-residue curves but find that they do not correspond with any theoretical curve, the steepness being much greater. The action of the H' and OH' cannot be one of simple control of the dissociation of the lysin, but they must also take part in the destruction by heat. It is impossible therefore from the data to determine the dissociation constants, but the I.P., where H' and OH' are in equal numbers, gives us a measure of the ratio of acid to basic constant.

From this investigation we can conclude that the experiments relating to heat destruction are in favour of the view that nasik haemolysin is an ampholyte. This is confirmed by the cataphoresis experiments. I must admit, however, that the presence of other proteins may influence the results. at any rate of the cataphoresis experiments. Similar objections may be raised to the whole series of the many determinations of the direction of movement in an electric field in the case of ferments, which have been investigated in the last few years, and it is quite possible that all these results will have to be revised.

SUMMARY.

1. The velocity of destruction of nasik haemolysin at 46.0° is at a minimum slightly on the acid side of the neutral point. This is in favour of the view that the lysin is an ampholyte with its acid dissociation constant greater than its basic, and the point of minimum destruction is the isoelectric point. The anion and cation are therefore thermolabile, and the undissociated molecules not so.

2. Cataphoresis experiments at 10° confirm this view.

3. Approximate values for the isoelectric points are

$$p_{H'} \text{ at } 10^{\circ} = 3.3; \quad p_{H'} \text{ at } 46^{\circ} = 6.1.$$

This decrease of acidity with rising temperature is also found in the case of simple ampholytes, but to a smaller extent.

4. Incidentally a criterion is given for the anomalous heat destruction of certain haemolysins, for instance, megatheriolysin, namely, that the process is not unimolecular at temperatures where the velocity of destruction decreases with rising temperature, but it can be shown to be so at temperatures considerably removed where the velocity of destruction follows the more ordinary course of increase with rise of temperature.

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absorption spectrum of this substance was accordingly determined, and it was found that absorption commenced at the line 2967 and extended to the extreme ultraviolet.

Now the line 2967 is the last line that permits the bacillus to grow when exposed to the influence of the complete spectrum as we have above pointed out. It appears then that that region of the spectrum which is responsible for the bactericidal effect is precisely that region that is absorbed by the antigen. It was still barely possible that there existed wave lengths that while being absorbed by the antigen were bactericidal but not capable of injuring the antigen. This was a bare possibility, but no more.

The experiment was accordingly made of exposing an emulsion of bacilli to the entire radiation of a quartz lamp through the (query) protective action of a solution of the antigen. Thirty rats were inoculated with a vaccine prepared in this manner, the exposure varying from five to 20 minutes. Subsequent test with a virulent culture, which killed all out of ten controls, also killed the whole of the 30 inoculated rats.

It is concluded from these experiments that the bactericidal and antigen injuring regions of the spectrum are identical¹.

I wish to express my profound sense of obligation to Dr C. J. Martin, Director of the Lister Institute, for his help throughout the entire work on the results of which these reports are based; also to my colleagues on the staff of the Lister Institute, most of whom have helped me in one way or another during the progress of the investigation.

¹ At this point Dr Rowland's investigations were interrupted by the European War.

LXXX. OBSERVATIONS ON THE LENGTH OF TIME THAT FLEAS (*CERATOPHYLLUS FASCIATUS*) CARRYING *BACILLUS PESTIS* IN THEIR ALIMENTARY CANALS ARE ABLE TO SURVIVE IN THE ABSENCE OF A HOST AND RETAIN THE POWER TO RE-INFECTION WITH PLAGUE.

By A. W. BACOT, *Entomologist, Lister Institute.*

VERBITZKI (1904) records the period for which he found fleas able to carry the bacilli and re-infect as three days.

The Commission for the Investigation of Plague in India (1906) found that in their experimental godowns, infection conveyed by fleas might take place 21 days after the flea population had had any opportunity of imbibing infected blood.

Bacot and Martin (1914) found that infected fleas which were regularly fed might live for 50 days at 10° C. to 15° C. and 23 days at 27° C. and remain infected at death.

Methods employed in the following Experiment.

Cages similar to those used in the Indian investigation (see Report, 1906, *Journal of Hygiene*, Vol. VI. p. 435) and also by Bacot and Martin (Report LXVII, p. 429, Plate XXIV, fig. 2, Plague Supplement, *Journal of Hygiene*, 1914), after sterilization, were prepared for the reception of mice and then stocked with 100 to 300 fleas (*Ceratophyllus fasciatus*) which had been infected by allowing them, when hungry, to feed on post-infected mice that were in the comatose condition that immediately precedes death¹. By delaying the fleas' opportunity to feed until this

¹ Three attempts were made to infect fleas (*Ceratophyllus fasciatus* and *Xenopsylla cheopis*), which had not fed previously, on mice that had already died of pest. In two of the three cases the fleas were placed with the mouse within an hour after its death. Subsequent dissection and the examination of stomach smears revealed neither blood nor lymph, nor was there any trace of bacilli, although in each instance the heart blood of the mouse showed a heavy septicaemia. Bugs (*Cimex lectularius*), which were tried at the same time, also gave a negative result as regards the presence of bacteria, but it is not possible to state definitely that they did not obtain any fluid at all from the bodies, owing to the fact that the gut of bugs which have been starved for considerable periods generally contains some remnants of a previous meal.

acute stage of the disease it was found possible to infect a much higher percentage than was otherwise possible. Samples of each batch were dissected and smears made from their stomachs showed on microscopic examination that from 65 % to 80 % of the sample fleas carried *B. pestis*.

After stocking the cages they were tested by placing two or three healthy mice in each. In all but two of the cases (Nos. 1 and 8) these mice died of typical pest. In the great majority of cases one or both of the glands on the groin were infected, less frequently one or both of the axillary glands were also involved, and, in very few cases, the axillary glands only were infected.

The spleen was in every case infected, usually heavily, and blood from the heart always showed some bacilli, the degree of septicaemia being generally marked.

As regards cages Nos. 1 and 8, no infection occurred within the usual period of three to five days, and the mice were removed and killed under ether. After the animals (dead or living) had been removed, the cages were covered with waxed cloth in order to check drying as far as possible, and then stored in a cool situation—the temperature varying from 35° F. to 60° F. with a mean of about 45° F. or 47° F.

When the desired period had elapsed the waxed cloth cover was removed and two healthy mice added to each cage.

The following table shows that cages Nos. 2, 3 and 8 remained infective for 29, 34 and 47 days respectively.

No. of Cage	Result of Preliminary Test	Period for which the Cages were stored without any host for the Fleas	Result
1	Mice not infected	18 days	—
2	4 mice died of pest	29 "	2 mice died of pest, 1 within 4 and 1 within 5 days
3	3 " "	34 "	1 mouse died of pest within 3 days
4	3 " "	35 "	—
5	3 " "	39 "	—
6	3 " "	45 "	—
7	3 " "	45 "	—
8	Mice not infected	47 "	1 mouse died of pest within 24 days
9	3 mice died of pest	59 "	—
10	1 mouse died of pest	68 "	—
11	1 rat and 1 mouse died of pest	73 "	—

In cages Nos. 2 and 3 it will be noted that the fleas had an opportunity of ingesting infected blood after the initial meal which infected them, but in cage No. 8 the only chance of feeding afforded them after the infecting meal was on healthy mice, which showed no signs of infection up to the time of their removal from the cage. The infection of the mice in cages 2 and 3 must have taken place within a few hours, at most, of the animals being placed in the cages after a lapse of 29 and 34 days respectively. In cage No. 8, however, there must have been an interval of about 20 days before the mouse was infected by the fleas in the cage. The records of the preliminary tests of the cages used in these experiments show that the period between the ingestion of bacilli by the fleas and the infection of a new host may be as short as three days—more usually it is longer, seven, nine or 12 days. It is of some interest to note in this connection that a period of latency in the development of plague among rats on shipping has been noticed by Dr C. Oswald Stallybrass, who, in writing me commenting on the subject, supplies the following instances: "Two cases have been brought to my notice within a month. One, a vessel from River Plate on which a small number of recently dead rats were found about five weeks after it had left the River Plate. In the second the ship had probably been infected not later than the 29th November, while five rats dead of plague were found in one limited portion of the ship on the 15th January—nearly seven weeks later. Two of the rats were recently dead and the other three less than ten days previously. Apart from two rats apparently killed by the dock labourers, no other dead or sick rats were found on the vessel, though 70 were destroyed by fumigation; these on examination proved to be healthy."

Conclusions.

1. Fleas (*Ceratophyllus fasciatus*) are able to carry *Bacillus pestis* for periods up to 47 days in the absence of any host and subsequently to infect a mouse.
2. That infected fleas, starved for 47 days and then placed upon a mouse, may not infect it for a further period of about 20 days.
3. There is no reason to suppose that the positive results obtained in these few experiments represent the limit of time after which infection may take place, but indicate that plague infection may persist in fleas for one or two months in cool weather and, subsequently, give rise to an epizootic.

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LXXXI. FURTHER NOTES ON THE MECHANISM OF THE TRANSMISSION OF PLAGUE BY FLEAS.

By A. W. BACOT, *Entomologist, Lister Institute.*

(With Plates XXXV and XXXVI and 2 Text-figures.)

SINCE the publication of the paper dealing with this subject (Bacot and Martin, 1914) further material in the form of longitudinal serial sections of infected fleas has passed through my hands. An examination of this material confirms the conclusions already arrived at, and adds one or two fresh details to our knowledge of the subject.

Text-figures 1 and 2 show the proventriculus in section and serve to illustrate the action of the opening and closing of the valve formed by the chitinized spine-like epithelial cells. When at rest the position of the spines and shape of the organ is shown in fig. 1—the opening into the stomach being free for the passage of blood. On the contraction of the muscular bands, which are arranged like the hoops of a barrel, the



Fig. 1.

girth of the proventriculus is constricted; the organ is by the same action elongated, and the lumen of the opening into the stomach is closed against the outward passage of blood (fig. 2).

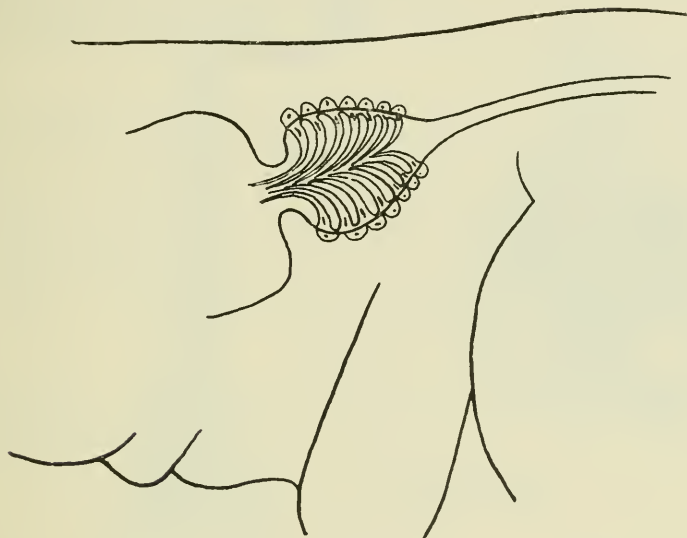


Fig. 2.

As already pointed out, it is the lodgment and growth of the bacilli among the spines of the proventriculus that is the initial stage of the blockage which during a subsequent feeding-act may lead to regurgitation of infected blood into the wound caused by the flea.

From the later sections it appears quite definite that in the first instance the block may be of a quite ephemeral nature—a second stratum forming in front of the first, both being so slight as to be depressed by the pressure of fresh blood entering through the oesophagus. When, finally, the process develops to the extreme stage shown in figure 4, Plate XXV, of the above-quoted paper, the condition is not necessarily fatal to the flea. The section of a specimen of *Ceratophyllus fasciatus*, Plate XXXV, fig. 1, shows the clearance of a passage through the centre of such a plug. The rupture of the obstructing mass does not, however, restore the lost valvular function to the proventriculus, but merely leaves a passage through which the blood can flow out of the stomach just as freely as it enters. The result, as is shown in this particular specimen, is that, after a full meal, blood, impregnated with bacteria, extends from the posterior portion of the stomach to the anterior chamber of the

pharyngeal pump. In this condition the infection of any animal on which the flea fed would seem to be more, rather than less, likely than in the case of a flea in which the proventriculus is completely blocked. With the existence of a patent proventricular valve the infected contents of the stomach may, owing to the peristaltic contraction of that organ, be regurgitated into the wound on the cessation of the suction of the pharyngeal pump.

Other sections show fresh unaltered blood in the sucking tube of blocked fleas, indicating that there is sufficient pressure in the distended oesophagus to prevent the normal clearance of the tube after a meal, which should take place owing to the action of the pump. One example shows the presence of a massive infection of such fresh blood along the course of the oesophagus to the pumping chamber, extending well down into the upper third of the sucking tube (see Plate XXXVI).

REFERENCE.

BACOT AND MARTIN (1914), Observations on the Mechanism of the Transmission of Plague by Fleas. *Plague Supplement III*, January 14, 1914, pp. 425—9.

DESCRIPTION OF PLATES.

PLATE XXXV.

Fig. 1 is a diagrammatic representation of a longitudinal section through the oesophagus (*oe.*), proventriculus (*p.*), and stomach (*s.*) of a heavily infected specimen of *Ceratophyllus fasciatus*. The light shaded portion shows where fresh blood, impregnated with free individuals of *B. pestis*, is present in the specimen, the darker shading indicates the solid mass of bacteria which has so far become disintegrated at its centre, as to be ruptured by the force of the blood pumped into the oesophagus, thus allowing the passage of blood to the stomach. The action of the valve is, however, inoperative, owing to the solidity of the mass of bacteria in which the spines of the proventriculus are embedded. $\times$ about 180.

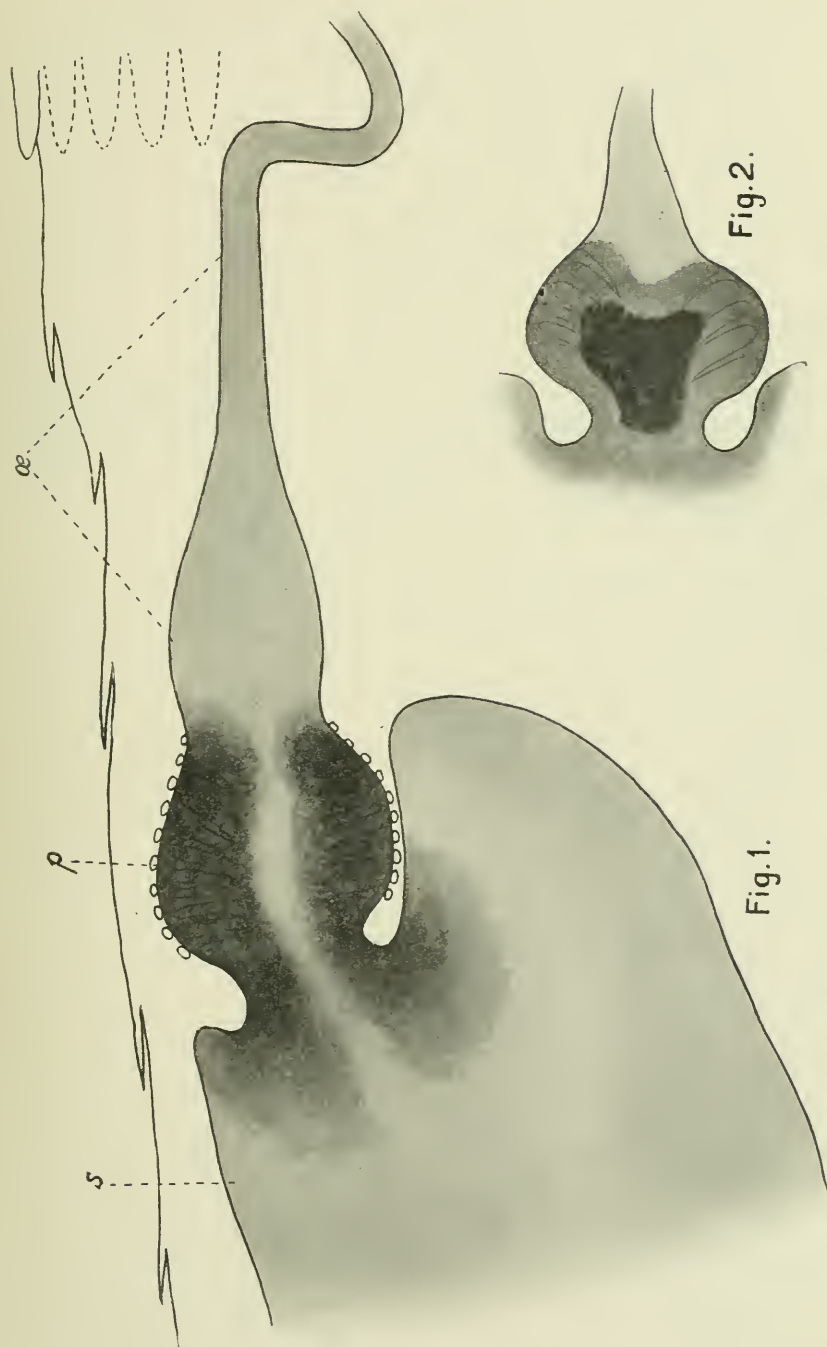
Fig. 2 shows a similar representation of a section through the dissected proventriculus and oesophagus of a specimen of *C. fasciatus*. It differs from fig. 1 in that the lumen of the valve is still obstructed by the disintegrating mass of an old plug and that the growth of bacteria surrounding this, which is of more recent growth, though yielding to the pressure of the fresh blood pumped into the oesophagus, has not yet been ruptured. $\times$ about 180.

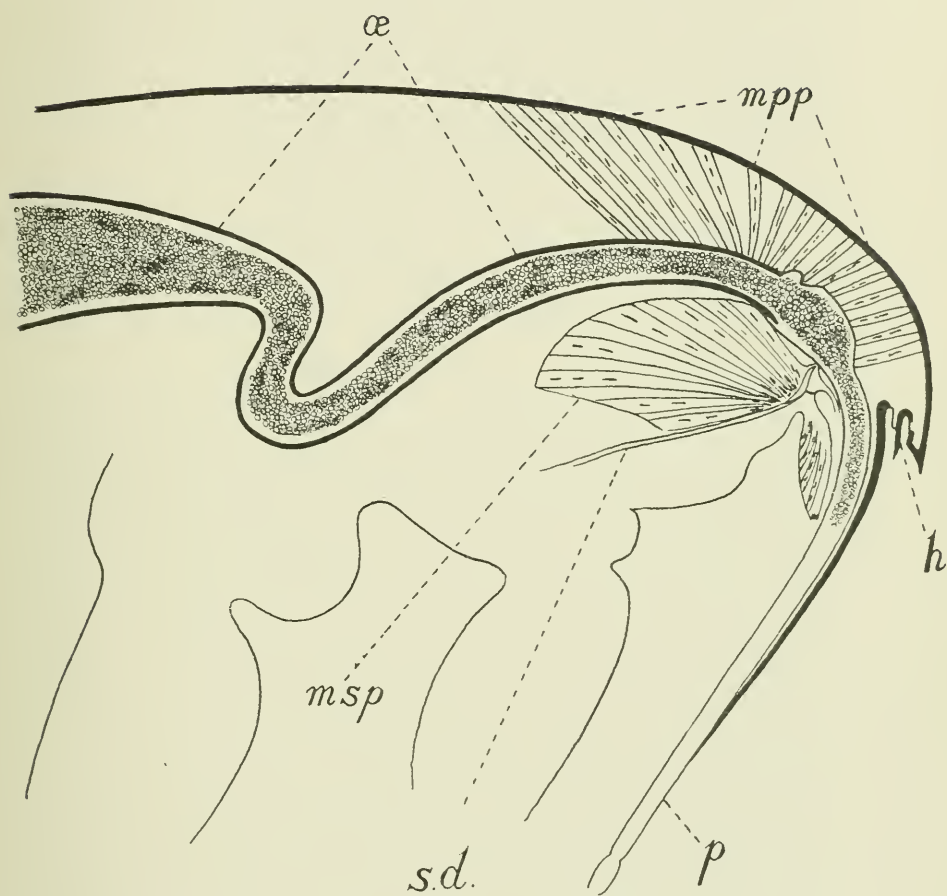
PLATE XXXVI.

Longitudinal section of head and thorax of a blocked specimen of *Ceratophyllus fasciatus*. $\times$ about 250.

h., arrangement of chitinous and soft integument in juxtaposition, forming a hinge which allows of the free ventro-dorsal movement of the sucking tube. *m.p.p.*, muscles of pharyngeal pump. *m.s.p.*, muscles of salivary pump. *oe.*, oesophagus containing a mixture of blood and bacilli. *p.*, proboscis or sucking tube. *s.d.*, salivary duct.

NOTE.—The bacilli are better seen with the aid of a reading lens.





LXXXII. NOTES ON THE DEVELOPMENT OF *BACILLUS PESTIS* IN BUGS (*CIMEX LECTULARIUS*) AND THEIR POWER TO CONVEY INFECTION.

By A. W. BACOT,

Entomologist to the Lister Institute of Preventive Medicine.

(With Plates XXXVII and XXXVIII and 1 Text-figure.)

Introduction.

VERJEITZKI (1904) was quite successful in his attempts to infect guinea-pigs by the bites of bugs (*Cimex lectularius*) which had previously been allowed to feed on animals that were dying of plague.

He found that allowing the insects to feed on the ear was more effective than letting them bite the leg; while, apart from situation, the virulence of the strain which killed the animals on which the bugs were fed was the crucial factor.

When the virulence was low there were no deaths, but, with a higher virulence, deaths occurred and the percentage of animals dying increased with the virulence of the strain of *B. pestis* used. Bugs that had been starved for longer or shorter periods were used, and it was discovered that the longer the period of starvation prior to the infected meal, the longer the period that the bacilli could be recovered from the bug. The longest time after infection that *B. pestis* could be recovered by culture was eight days, but the longest period after infection that the disease could be conveyed by bite was five days. The smallest number of infected bugs used successfully to convey infection was three. The same batch of infected bugs was able to transmit the disease to two batches of animals on successive days, but failed on the third occasion.

Nuttall (1899—1900) tried on four occasions to convey the disease to mice by means of infected bugs, but failed on each occasion. He was able to recover *B. pestis* from the body of bugs five days after the infected meal.

Jordansky and Klodnitsky (1908) found that attempts to convey plague by allowing bugs to feed on a mouse sick of the disease, and before they were satisfied removing them to a healthy mouse, failed. They noted that the number of bacilli in the bug's stomach increased from the third to the sixth day after the infected meal. On the tenth day involution forms appeared, and subsequently, although the bacilli disappeared from view, they were recoverable by culture after 35 days.

Jordansky and Klodnitsky (1910) stated that, of 13 bugs fed on a pest infected mouse, two survived for 83 days and were then fed on a healthy animal. Five days later *B. pestis* was demonstrated in the bugs both by microscope and culture.

The following notes are the outcome of some rather desultory work that was performed as time and opportunity permitted during the course of a more systematic research concerning the transference of pest by fleas.

Methods.

Bugs (*Cimex lectularius*) were infected by allowing them to feed on mice that were in the comatose condition that immediately precedes death from plague. In attempting to reconvey the disease to mice by allowing the bugs to feed on them, it was found that active healthy mice eat the bugs unless some provision is made to afford the insects cover from their attacks. Mice can of course be bound, but they are restless in this condition and it was especially desired to allow the bugs a chance of undisturbed feeding while the mice slept. The usual points of attack are apparently the ears, tail or feet. A simple method of overcoming the difficulty is to bore a one inch hole in a wooden block some three or more inches long and then to make a number of saw cuts in the block so as to penetrate into the hole. A suitable tube can be arranged by cutting the bases off two solid wooden postal blocks, such as are used for despatching specimen tubes, and then nailing them together at right angles (fig. 1).

Unfortunately this plan was not devised until after such infection experiments as the time at my disposal allowed for, had been carried out. The method was found to be successful, however, in so far as by its adoption it was found possible for bugs to be kept with active mice in a cage suitable for infection experiments, and to feed freely upon them.

The effect of infected blood upon the bugs.

Bugs in their first instar as well as older larvae, nymphs and adults, were infected. The infected blood was the apparent cause of considerable mortality among the insects; especially was this the case with the first instar larvae¹.

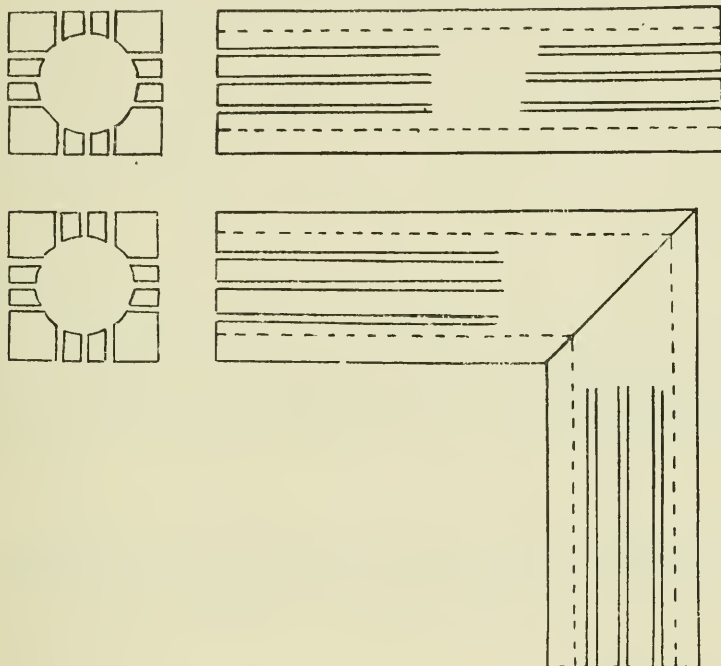


Fig. 1.

Twenty young bugs that had hatched a week or two previously were allowed to make their first meal on an infected mouse. Most of them fed to their full extent. After feeding they were placed in an entomological glass-bottomed card box and kept in a dry cupboard at 60—69° F. Two, which appeared to be injured, died the day following their meal,

¹ To avoid any confusion I must explain that the conditions under which bugs are kept after a full meal are important, even if the food be normal human blood. Placed at the bottom of a glass tube in a cool, moist situation, there was considerable mortality in my experience, whereas control insects kept in card boxes in a dryer and warmer situation showed no deaths. Young (first instar) bugs are more delicate in this respect than older larvae, nymphs or adults.

and an examination of the blood in their stomachs showed that it was heavily infected with *B. pestis*.

Three moulted four days after feeding, and then died; thirteen which had not moulted after seven days, were found lying at the bottom of the box, either recently dead or paralyzed. The two remaining did not moult, and were still active on the eighth day, but were found in the same paralyzed condition as the others on the tenth day.

As regards external appearances, the only unusual feature noticeable in these young bugs and the older ones which died in a similar paralyzed condition, after full meals of infected blood, was their failure to reduce their bulk, a process which is normally noticeable in healthy specimens within a few hours after a full meal. In this respect their symptoms are similar to those which accompany the death of normally fed bugs which have been kept in too cool and moist a situation after a full meal.

Smears of the stomach contents of infected bugs made at various periods after the infected meal show the following appearances. In the majority of the cases the specimens were paralyzed or dying at the time they were dissected. In only a few instances were healthy or definitely dead insects used, but the actual condition of the insects at the time of making the preparation made no apparent difference.

STOMACH CONTENTS TREATED AS BLOOD SMEARS.

(Stains used—Leishman or Carbol-Thionin.)

Period and temperature
between infecting meal
and death of bug

Remarks

1 day at about 65° F.

Some large bipolar staining bacilli present, but the infection is chiefly of the "Yeast" like forms photographed by Dr Rowland (1914) in his recent paper on "The Morphology of the Plague Bacillus," figs. 16 and 17. The organisms are generally scattered in the smear—there is no clustering. Phagocytes intact and numerous. (1 specimen.)

3 days at about 80° F.

A very dense growth of the small culture form (see figs. 1 and 2 in Rowland's paper). No "Yeast" forms, a few large forms and many smaller ones show bipolar staining; a tendency to cluster and clump is noticeable. There are but few phagocytes visible. (2 specimens.)

3 days at 60° F.

Growth much less dense than in the last—mostly consisting of the “Yeast” form of the small culture form; a few large bacilli, which show bipolar staining. No clustering. A slight tendency to form chains, but this does not progress beyond threes and fours of the slender type, phagocytes not numerous. (1 specimen.)

A second specimen shows a denser growth with the phagocytes more numerous. A third agrees with the second, but has a rather more varied assortment of form, and fewer of the “Yeast” form.

5 days at 65° F.

This differs but little from the 3-day specimens, but there is no trace of any “chain” tendency.

5 days at 32° F.

Bacteria few and scattered; all large form, often short and mostly showing bipolar staining; blood appears unaltered in structure. (1 specimen.)

5 days at 80° F.

Very numerous “Yeast” and short, broad bipolar staining types; a few very long and broad and a few long and narrow, both bipolar staining. A very noticeable feature are the clumps of unstainable material, presumably of autolyzed blood and unstainable bacteria. No clustering of the staining forms. (1 specimen.)

7 days at 65° F.

Bacteria very numerous, scattered, of the “Yeast” type, with a certain admixture of large, short bipolar staining forms, and in one specimen a small proportion of the slender bipolar forms also. Phagocytes are present, but the smears are too poor to allow of any decision as to their condition. (4 specimens.)

8 days at 60—65° F.

Very numerous, the greater portion of the “Yeast” form; a few pairs of the slender forms set end to end, but no actual chains. There is also a sprinkling of the large bipolar staining forms, mostly rather stumpy. Same tendency to clustering is noticeable, but no actual clumps. Phagocytes are present. (4 specimens.)

10 days at 60—65° F.

Large clumps composed of autolyzed blood, and masses of the bodies of bacteria which do not stain are present. Mixed with them in the clumps, and also generally distributed throughout the smear, are numerous well-stained forms, mostly of the “Yeast” type. There is also a sprinkling of large bipolar staining bacilli, for the most part rather short. The general appearance reminds one of some of the phases found in fleas, with the modification that recognizable phagocytes are present after ten days. (1 specimen.)

Period and temperature
between infecting meal
and death of bug

Remarks

10 days at 60—65° F.

A second specimen shows neither the clumps of autolyzed blood nor the non-staining bacteria, but only a general distribution of numerous "Yeast" forms and some other types. The general appearance is very different from the last. The blood has apparently been but little altered—red blood cells and phagocytes being present in numbers. (1 specimen.)

15 days at 60° F.

This bug had only had a small meal. The stomach contents mostly consist of unsuitable material—clusters of the non-staining bacteria being mixed with the autolyzed blood. A very few and widely scattered stained forms are present and some of these show bipolar staining. No phagocytes discernible. (1 specimen.)

15 days at 65—70° F.

Various small faintly staining forms are present, mostly short; in many cases showing bipolar staining. A few pairs visible; recognizable phagocytes rare. (1 specimen.)

21 days at 28—30° F.

Large bipolar forms, many dividing and a few short chains. The bacilli few and scattered; the blood apparently unaltered structurally. (1 specimen.)

21 days at 65° F.

Numerous but scattered, for the most part small, inclining to the "Yeast" form, but some bipolars of small size are present. Phagocytes still easily recognizable.

60 days at 80° F.

The smear shows masses of small coccus-shaped bodies embedded in the autolyzed matter of which the smear is made. These bodies are not very sharp in outline and are usually unstained and of a dull greenish hue. I have, however, come across one or two small groups of similar bodies which are faintly stained. When compared with definite pest smears, one hesitates, in spite of the wide variation of *B. pestis*, to say that these minute and indistinct bodies are pest bacteria, nevertheless I suspect this to be the case and look upon them as the dead or degenerate remains of a starved culture. (1 specimen.) In this connection note the following example.

A bug that had fed on a mouse dying of pest was then fed at intervals of 3, 7, 18 and 19 days on a normal rat, which did not contract the disease. The bug died on the 40th day after the infected meals. The bacteria present in the stomach smear are very numerous and much smaller than those in the other smears, in some cases they are as minute as the bodies in the last specimen referred to above. In shape they are mostly of

either the "Culture" or "Yeast" forms, but others still, if unusually small, are of the slender type arranged in twos and threes. A few of the normal blood forms showing bipolar staining are present—these also being very small in comparison with similar forms on other smears. All of these types stain more heavily than usual. Red blood cells and phagocytes are numerous and easily discernible, but the former appear in poor condition—being much distorted in shape.

It will be seen that the characteristic form of *B. pestis* in these smears is that which Rowland (1914) has termed "Yeast" like forms and of which he shows two photographs, figs. 16 and 17. Speaking from memory, I do not recollect to have found this form in preparations of the fleas' stomachs, certainly it was never a distinctive feature as it is in the bug. As was to be expected, the number of bacteria present is to a considerable extent dependent on the period and temperature between the infected meal and the death of the bug. But, apart from this general result, to which there are some exceptions, it does not seem possible to draw any very definite conclusion as to the relationship between temperature or period and the type of *B. pestis* present or the development of the growth. There is, however, as in the flea, a definite association between the presence of unstainable bacteria and dark autolyzed material; also a suggestion that agglomerated growth may be associated with the destruction of the blood cells.

A clear, colourless fluid present in the rectum of many of the first instar bugs which were killed or paralyzed as the result of the infected meal, was squeezed out prior to the rupture of the crop. This showed no trace of *B. pestis* on microscopic examination.

Sections.

The bugs, after fixation, were embedded in clove oil celloidin and paraffin (an adaptation of Entz's method, *Arch. Protistenk.*, xv. 1909, p. 98), and then stained with Haematoxylin and Eosin for tissues and blood, subsequently with Carbol-Thionin for the bacilli.

SECTIONS OF PEST INFECTED BUGS.

(Stained Haematoxylin, Eosin and Carbol-thionin.)

Period and temperature
between infecting meal
and death of bug

Remarks

1 day at 60—65° F. (Pl. XXXVII, fig. 1.)

Transverse about the middle of crop (bug in first instar), $\times 180$. The infection is not generally diffused; it appears to be of a stratified nature, but with many small detached colonies. The dorsal area is decidedly freer than the ventral, suggesting a gravitational effect which is not, however, consistently borne out, as there is one large wave of infected blood in the dorsal area which comes in contact with the upper wall of the crop. There is a very definite association of the phagocytes in the blood with the bacteria, quite apart from any gravitational process. The growth of the bacilli must have been very rapid to have progressed to such an extent in 24 hours. Many of the sections show curiously sharp lines of demarcation between the masses of infected and uninfected blood. A belt of normal blood of remarkably even thickness, coming between a less regular one, carrying a mass of bacteria and phagocytes, and the wall of the crop may be observed. The division is as sharp as if a line had been drawn.

With a $1/12''$ oil immersion lens (Pl. XXXVII, fig. 2) the bacilli are seen to be all of the culture form and the higher magnification also reveals numerous darkly staining bodies of varying sizes, larger than a bacterium and smaller than a red blood cell. From the almost invariable association of these particles with infected areas where phagocytes are present, it would seem definite that they are not normal to the blood or fluids of the bug's stomach. Appearances suggest that they are fragments of the nuclei of dead phagocytes.

2 days at 60—65° F. (Plate XXXVIII.)

Longitudinal vertical section of adult bug. The crop much distended with blood, $\times 180$. The central area shows very numerous patches of a dirty brown colour which contrast sharply with the eosin stained blood. These patches vary greatly in both size and shape; towards the anterior end of the crop is a very large patch of this description. The central area of this patch is fragmentary and loose in character, but towards its periphery it becomes very dense and within this dense belt large numbers of phagocytes are massed together. At its outer margin there is a more or less broken brightly staining fringe.

With the $1/12''$ oil immersion lens (Pl. XXXVII, fig. 3) the dark patches above referred to are all found to be very similar in character, consisting of masses of small unstainable bodies which by their number and agreement in shape I take to be bacteria which do not take the stain, and fragments of varied, usually large size and irregular shaped (presumably autolyzed) blood cells or fragments of them. At the periphery of these masses there is a more or less broken fringe of stained bacilli. There

are also occasional, small patches of stained bacilli among the red blood cells quite apart from the masses of autolyzed material. The phagocytes are almost invariably confined within the autolyzed areas—the larger and presumably less recently developed being disproportionately favoured in this respect; in many instances they appear distorted and fragmentary. A further difference between the very large and the smaller patches is that the former alone show a degenerating central area. The same phenomena, mentioned in the account of the first specimen, of sharp lines of demarcation between infected and uninfected areas is apparent in this specimen as well. The appearance suggests that masses of bacteria had developed against the wall of the crop until they had attained some cohesion and then becoming shifted, uninfected blood had flowed in between them and the walls of the crop. In this specimen blood is present in the oesophagus from its entrance into the crop forward to the posterior end of the pump. A few stained bacteria are to be observed in the tube in front of its passage through the brain.

5 days at 60—65° F.

Longitudinal vertical section of adult bug, $\times 180$. The development in this specimen differs very greatly from that in the last. The red cells are very distinct, but few if any phagocytes are present in the crop, while in place of the stratified appearance after the one-day infection or the large autolyzed clumps in the last specimen, there is a much more even distribution of small patches of stained bacilli with slender ramifications. The autolyzed material consists of generally distributed specks. The slender portions of the stomach behind the crop and the gut, however, which were more or less empty in the other specimens, are in this one distended with partially digested material. Large numbers of red blood cells, more or less perfect in outline, are present in both tubes, a few only of which have taken the stain, the others are mere outlines of the same tint as the remainder of the autolyzed material.

With a $1/12''$ immersion lens it is seen that this unstained material consists of what are probably fragments of blood cells and masses of unstained bacteria, with a few well-stained examples among them. In smaller areas the material present consists of masses of stained bacilli with a smaller admixture of unstainable ones. A few well-stained but distorted or fragmentary phagocytes are also visible in the lower stomach.

The difference between this and the last specimen in fact amounts to the segregation of the phagocytes and larger masses of autolyzed material to the lower portion of the stomach and gut on the one hand and their general distribution in the central area of the crop on the other.

It seems not impossible that such a result might follow the withdrawal of blood from the crop to the lower stomach, as no doubt the central area would respond to suction more readily than the outer layers, but the completeness of the segregation of the leucocytes arrived at in this specimen seems hardly credible by these means alone.

This specimen also shows blood in the oesophagus and the posterior end of the pumping chamber. There are a few bacilli to be found free in this

Period and temperature
between infecting meal
and death of bug

Remarks

blood, while in the folds and puckers formed by the walls of the oesophagus at its junction with the pump, dense clusters are to be seen from which small detached groups of bacilli are passing out through the narrow entrance into the pump.

8 days at 60—65° F.

Longitudinal vertical section of a first instar bug; $\times 180$. The development in this specimen consists chiefly of a large infected area within the crop, in addition to which there are some streaks ramifying from this centre with small detached patches. The lower, tubular portion of the stomach contains large masses of partially digested blood, which has not stained, while the contents of the crop at the posterior angle look dense and are of a yellowish colour. The leucocytes are as usual almost all crowded into the infected area.

With the $1/12''$ immersion lens the bacteria are seen to be clustered within the central area and other infected patches, and to stain vividly. The same dark staining spots previously described in the account of the first specimen (1 day) are present within the large infected area. Their appearance in this specimen bears out the previously made suggestion that they are fragments of the nuclei of the leucocytes. The yellowish area at the extremity of the crop seems to consist of clotted blood; it is very dense and shows no structure, while the bacilli are thickly and evenly distributed, giving the patch a granular appearance.

The autolyzed material in the lower portion of the stomach is seen to consist of more or less digested blood, mixed with large clumps of unstained bacteria.

A second specimen, *cut horizontally*, shows a much weaker infection in the crop, but the posterior portion of the stomach, or a fold of the intestine is closely packed with a dense mass of vividly stained bacilli, the unstained autolyzed material being confined to a small central portion of the tube in this section. This is by far the densest and, presumably, the most rapid growth I have seen in the bug and approaches more nearly to that which occurs in the laked blood within the stomach of the flea than any of the specimens cut as sections have yet shown.

A third specimen, *cut transversely*, agrees pretty closely with the 1-day specimen as regards the development within the crop, but the tubular portion of the alimentary system is seen to be well filled with unstained material. With the $1/12''$ objective this latter is seen to consist as usual of more or less broken down blood cells and dense masses of unstained bacteria.

A fourth specimen, *cut in longitudinal vertical direction*, agrees closely with the last in respect of the development of the infecting growth. It also shows blood to be present in the pumping chamber, which is heavily infected in places.

34 days at 60—65° F.

Longitudinal vertical section of a nymph. All the blood in the crop has been broken down, and there are no signs of bacilli in it. The autolyzed material in the tubular portion of the alimentary system contains numbers of small specks which differ considerably from the appearance of the unstained bacteria which give a granular appearance in the definitely infected bugs. There is, of course, a possibility that they may be connected with a previous growth of bacteria, but I have seen very similar objects among the autolyzed material in the intestines of normally fed bugs, and am of opinion that this insect was not infected at the time of death.

74 days at 60—65° F.

Longitudinal vertical section of adult. There is no definite evidence that this specimen was infected when it died. The autolyzed contents of the alimentary canal show numbers of the small unstained bodies which are possibly the remnants of bacteria, but beyond the fact that their size and distribution are more favourable to this view than in the last (34-day specimen) it is not possible to go with safety.

The sections do not give such clear details of the character of the individual bacilli as do smears, for the following reasons, among others: (1) Dehydration causes the bacteria to shrink whereas, in a smear, shrinkage is prevented; (2) The sections are rather thick (about 5 or 6 μ); (3) The bacilli are thickly clustered together; (4) The staining, Haematoxylin and Thionin, is not very distinctive.

In all but the last two specimens dealt with (34 days and 74 days) the presence of unstained bacteria among the autolyzed material in the gut can be definitely determined, but with regard to these last there is considerable doubt, as the small dark bodies present approach more nearly to those seen among the detritus in the intestines of normally fed specimens of *Cimex lectularius* and *hirundinis*.

The points of most general interest appear to be the very definite association between the leucocytes and the bacteria. That this is due to the initiative of the leucocytes after the blood has been ingested, would seem to follow from the fact that they are massed within the largest and, presumably, oldest colonies, out of all proportion to the entire area infected; that is taking the smaller and scattered masses of bacilli into account as well as the large masses which are usually very few in comparison with the former.

Another interesting feature is the striking differences of development that generally obtain in the bug from that found in the flea. In the former, growth arises and tends to continue as isolated colonies, while in the flea the growth speedily loses its localized form and becomes general, often filling the entire stomach with a cohesive mass. It seems probable that this divergence is due to the speedy breaking down of the blood which takes place in the stomach of the flea, while in the bug the cellular structure of the blood would appear to be present for days as against a fraction of an hour or so in the flea. As regards the various phases of development exhibited by different bugs, this may be in large measure due to the volitional character of the bug's digestive apparatus. Verjbitzki called attention to the fact that starved bugs gave a different reaction to the more recently fed specimens; the implication being that the starved insects were the more thrifty in the use of the blood that they had ingested.

The general appearance of the specimens suggests that growth is at first rapid, but afterwards slows down and loses its vigour as it spreads outwards, leaving an exhausted area within the growing fringe, much as the small toadstools which form "fairy rings" in the turf are stated to do.

There are curious divergences in the development of the growth of colonies even in specimens of the same age which were fed on the same animal, kept under identical conditions for the same period and then fixed and sectioned under at one and the same time. Such differences must seemingly rest with the bug itself and are probably due to variations in the digestive action of the different specimens.

Cases of infection following the bites of bugs carrying B. pestis.

Only on two occasions did death follow from the bite of infected bugs and there was one doubtful case of infection when a rat fell sick and showed some of the symptoms of an animal suffering from pest, but it subsequently recovered. .

A number of trials were made with two separate batches of infected bugs each consisting of larvae, nymphs and adults. The insects were kept in glass tubes and allowed to feed through a gauze covering on the shaved abdomen of the rat—the gauze cover being changed before each experiment. A number of rats were tried and the insects were in most cases allowed to feed on the same rat on several occasions. The result, as stated above, was one death, and one possibly infected rat which did not die, out of seven or eight rats.

The other experiment which gave a positive result was with a mouse. A batch of bugs, consisting of well-grown larvae, some nymphs and several adults were fed on a mouse just prior to its death from pest. They were then kept in a card-sided glass-bottomed box at about 60—65° F. without further opportunities of feeding. From this stock were drawn a number of the examples already discussed as smears or sections.

The survivors died off gradually until on the forty-eighth day there were only five living and one of these was very feeble. These five survivors were placed in a deep glass jar with some crumpled filter paper to afford them foothold and cover and a healthy mouse was put in. On the following morning only one of the bugs could be found, the others must have been eaten by the mouse, but the sole survivor had managed to feed.

Five days later the mouse died of typical pest. The glands of the right groin and axillae were both infected, while smears of the spleen and heart blood also contained numbers of *B. pestis*. Mice inoculated with the organisms recovered from the liver of this specimen also succumbed to the disease. Although a number of attempts were made to feed the surviving bug on mice and the ear of a guinea-pig, it could not be induced to bite and was killed when very feeble 74 days after its infected meal¹.

Discussion of the possible mechanism of infection by bugs.

The marked difference in the development of *B. pestis* in fleas and bugs is probably due to the wide divergence in their structure and habits. The digestive processes of the flea result in a rapid destruction of both red blood cells and leucocytes, leaving the contents of its stomach very much in the condition of an artificial culture media, if we may judge from the development of the bacilli. In the bug, on the other hand, the crop would seem to serve chiefly as a reservoir for the storage of food: the blood within it remaining, so far as structural appearances go, unaltered for days after its ingestion. Sections show this organ to act mainly as a simple walled sack. At its posterior end only does the development of the epithelial cells suggest that any digestive process is possible. Whether as a consequence or as an accompanying phenomenon, the conditions within the crop of the bug do not favour the same comprehensive development of *B. pestis*, which is normal in the flea.

¹ See note under "Sections" of the last specimen which was killed and cut 74 days after the infecting meal.

Behind the crop in the long tubular portion of the stomach which continues back to the malpighian tubes, digestion is very evident and in this area occasionally a more comprehensive growth takes place. As a rule, however, the digestive process seems powerful enough and sufficiently rapid to prevent any large area being occupied by an evenly staining growth of *B. pestis*.

As regards structure there is a very marked difference between fleas and bugs as to the junction of the oesophagus and stomach. Bacot and Martin (1914) show that the blocking of the intricate valvular proventriculus of the flea is a crucial factor in the mouth transmission of plague by these insects. With the bug the likelihood of any such stoppage is improbable. The large flat head of the *Cimex lectularius* accommodates a much more powerful pump than does the narrow laterally compressed head of the flea. It follows that the relative ratio of the pump to oesophagus in cross section is very different in the two insects, the dimensions of pumping chamber in the bug being very many times that of the short tubular oesophagus through which the blood passes into the crop. In conjunction with the alimentary system of the flea that of the bug might be described as valveless. Actually the oesophagus, as seen in sections, is found to be thick-walled with a folded and puckered inner lining which greatly obstructs the lumen of the tube. The appearance premises great capabilities of distension or extension under stress of circumstances, but little in the way of effective control of any inward or outward flow of blood, while I have been unable to trace any muscles or muscular attachments which appear capable of any constrictive power. The action of the crop itself, however, as it fills and distends in all directions will not only allow of the shortening of the oesophagus, but may finally, as it pushes forwards, cause the oesophagus to take up a more or less transverse or even a folded position which may be sufficiently acute to close the tube. Although the arrangement appears to be shipshod in the last degree, the pressure of the blood within the crop possibly proves effectual enough in preventing egress of the contents of the crop in practice. If it is less perfect from a mechanical point of view than the valve of the flea, there is little danger of any growth of *B. pestis* being able to effect a stoppage.

It has already been noted in the descriptions of the sections that *B. pestis* does actually multiply among the folds of the lining membrane of the oesophagus, and in one specimen small groups were to be seen in the act of passing out of the narrow entrance into the pumping chamber. The chances of any direct regurgitation of infected blood from the crop

into the wound made by the bug when feeding are lessened owing to the disparity in size between the oesophagus and the pumping chamber, as it is necessary that the latter be filled before any regurgitation can take place.

We must, however, bear in mind the fact that when a bug is disturbed during its meal that it will feed again within a short interval, and it seems not impossible that injected blood might at the moment of interruption be regurgitated through the pump and washed down into the wound at the next attempt by the flow of saliva which enters into the pharynx anterior to the pump. If such regurgitation takes place the loose character of the colonies within the crop in conjunction with the efficient mixing apparatus afforded by a powerful pump jerking fluid through a narrow tube into an elastic bag would seem to be an ideal aid to the chances of a sample of any bacteria present in the crop being mixed with the regurgitated fluid.

This explanation is, of course, mere speculation, but there seems to be no doubt that the infection which followed Verjbitzky's experiments was due to regurgitation.

In the cases I have recorded above one may possibly have arisen owing to injury to a bug by the mouse while it was feeding, but in the case of the rat it must have been due to simple regurgitation.

Rectal infection may, I think, be safely dismissed, owing to the fact that bugs, as pointed out by Martin (1913), do not defaecate during meals, as fleas are occasionally known to do, but hurry after their meal to some nook or cranny to digest at leisure.

Conclusions.

(1) That for a percentage of bugs (*Cimex lectularius*) and probably all newly hatched ones, a meal of septicaemic blood from a mouse dying of plague is fatal.

(2) Bugs which are not killed by the infecting meal are capable of carrying *B. pestis* and reinfesting mice after a period of 48 days' starvation.

(3) The development of *B. pestis* within the crop of bugs differs generally from that which takes place in the stomach of the flea in respect of its slower and looser growth, this limitation of activity being accompanied by and possibly due to the preservation of the structural character of the blood for many days after its ingestion into the crop.

(4) The absence of any definite valve between the pump and the crop, together with the looser nature of the growth within the bug, preclude the idea of such mechanical blockage as causes regurgitation and mouth infection by fleas. It may be surmised, however, that mouth infection when not caused by accidental or other injury to the bug while feeding, may be due to interruption followed by a second attempt.

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EXPLANATION OF PLATES.

Plate XXXVII. Fig. 1. Transverse section through middle of crop of bug (*Cimex lectularius*) in first instar. Plague infection. 1 day at 60—65° F. *a.* Wall of crop. *b.* Infected blood. *c.* Uninfected blood. *d.* Phagocytes. ($\times 180$.)

Fig. 2. Portion of Plate XXXVII, fig. 1. *a.* Wall of crop. *b.* Blood infected with plague bacilli. *c.* Uninfected blood. *d.* Phagocytes. ($\times 1000$.)

Fig. 3. Portion of Plate XXXVIII. *a.* Autolyzed material containing bacilli. *b.* Phagocytes. *c.* Uninfected blood. ($\times 1000$.)

Plate XXXVIII. Longitudinal vertical section of adult bug (*Cimex lectularius*). Plague infection. 2 days at 60—65° F. *a.* Integument. *b.* Pharyngeal muscles. *c.* Pharyngeal pump. *d.* Oesophagus filled with blood. *e.* Crop. *f.* Ant. cerebral ganglion. *g.* Post. cerebral ganglion. *h.* Ventral nerve cord. *i.* Phagocytes in autolyzed blood. ($\times 135$.)

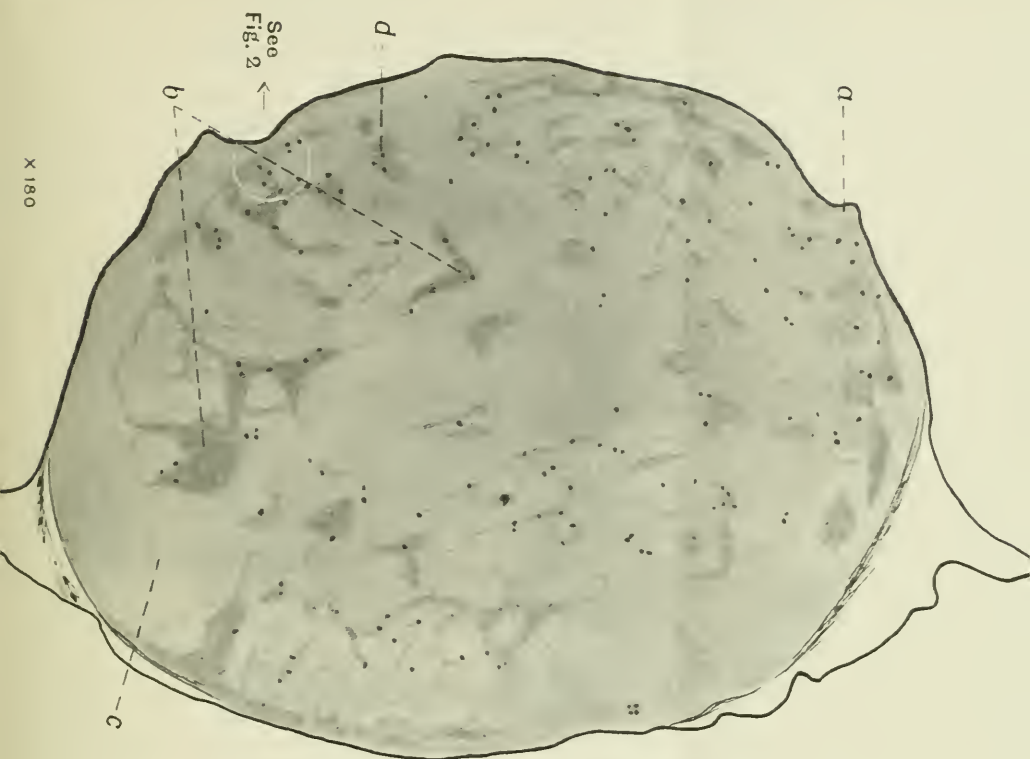


Fig. 1.

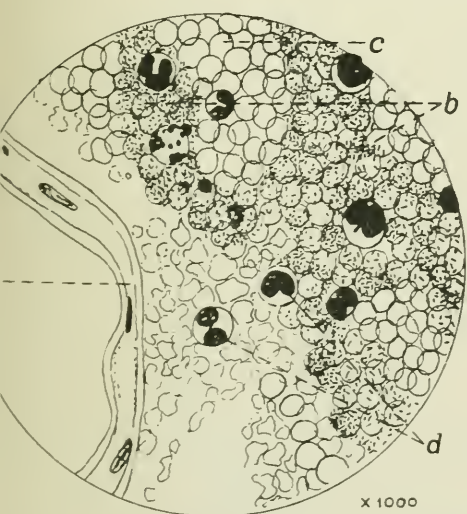


Fig. 2.

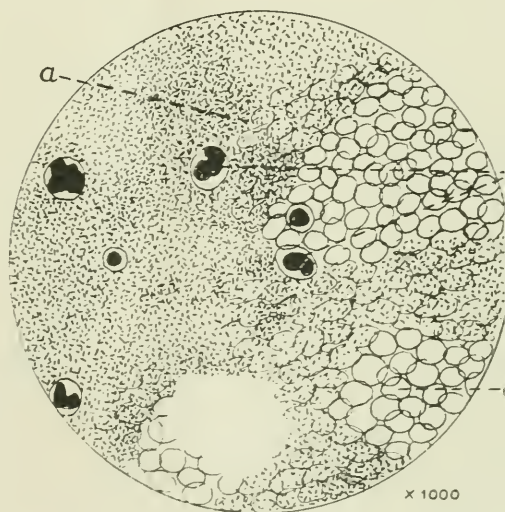


Fig. 3.

(4) The absence of crop, together with the preclusion of the idea of infection through the mouth and mouth infection by the bug while feeding, attempt.

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Plate XXXVII. Fig. 1
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b. Infected blood. c

Fig. 2. Portion
with plague bacilli.

Fig. 3. Portion
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Plate XXXVIII. Longitu
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THE FLEAS FOUND ON RATS AND OTHER
RODENTS, LIVING IN ASSOCIATION WITH
MAN, AND TRAPPED IN THE TOWNS, VIL-
LAGES AND NILE BOATS OF UPPER EGYPT.

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Description of Methods of Collection.

THE collections of fleas described in this paper were made during an enquiry into the plague conditions of Upper Egypt, carried out in 1912 and 1913 on behalf of the Egyptian Government. With few exceptions the fleas were taken from rats or other rodents trapped either in the native houses or in the Nile boats (native river craft), which were infested with rats.

The method of trapping was as follows: The trap used was the ordinary wire cage trap with two compartments separated by a partition into which fits a hinged platform with counterweight permitting ingress of the rats, but preventing their escape. The traps containing catches, before being brought from the houses to the laboratory, were enclosed in a black cloth bag to lessen the risk of fleas escaping. At the laboratory the bags were removed and the traps were placed in suitable boxes on the bottom of which were removable metal trays covered with white wax-cloth. Chloroform was poured into the boxes in order to kill the rats and fleas. A considerable number of the fleas were found to have dropped from the rats on to the white cloth, and thus could be easily seen and collected. The chloroformed rats were thoroughly searched for fleas upon a table covered with white cloth.

The most extensive collection in the series is that from Assiut (245 miles south of Cairo), for the reason that in this town trapping operations were carried out continuously during two years. With the idea that the flea population might show seasonal variations as regards species or proportions of species, collections of consecutive periods of two or three months were kept separate, and samples from each lot have been examined, without, however, revealing any seasonal difference.

Comments on the Localities from which the Fleas were collected.

The localities represented extend from Cairo to Komombo, an agricultural estate 460 miles south of Cairo, and 26 miles north of Assuan, and are indicated upon the sketch map in the text (p. 500).

With the exception of Cairo and Komombo the towns and villages from which fleas were collected may be regarded as typical for Upper Egypt in respect of the housing conditions. Most of the houses in an Upper Egyptian town or village are constructed of burnt bricks held together chiefly with clay (Nile mud), in which, when dry, rats make their burrows. The earth foundations of the walls are also riddled with rat burrows. As a result rodents infesting houses are in close association with the human inhabitants and with their domestic animals, *e.g.*, dogs, cats and fowls.

The conditions in the quarters in Cairo which yielded the fleas in our collections differ from the above description. The quarters trapped were the Mousky—the native bazaar quarter in the centre of the city, and Boulac, a quarter adjacent to the Nile and opposite to the island of Ghezireh. The houses in these quarters and in Cairo generally are more substantial than those in Upper Egypt, many of them being built of limestone from the neighbouring hills.

From the standpoint of rodent and flea infestation Komombo is of considerable interest. The Komombo estate is a cultivated area of 20,000 feddans (acres), scattered over which are a number (29) of small hamlets each with an average population of about 500 persons. The houses are built, some of stone, others simply of dried mud. The whole estate is of very recent origin, having been developed by means of irrigation from purely desert land only some ten or twelve years ago. *Arvicanthus niloticus* and *Mus norvegicus* are found in the houses and in the fields, but *Mus rattus* and *Acomys cahirinus* have not been met with.



The Nile sailing boats (feluccas) are built with inner and outer planking bolted to the cross-ribs, thus providing a hollow space in which the rats live. These boats carry cargo—grain, coal, etc.—but also passengers, and ply up and down the Nile from Rosetta and Damietta to Assuan. The rodent infestation on board consists chiefly of *Mus norvegicus*, differing in this respect from the rodent population ashore (Komombo excepted), where this species is, in our experience, a rare inhabitant of houses.

Meteorological data for Cairo, Assiut and Assuan are appended.

TABLE I¹.

Mean monthly meteorological observations recorded at 8 a.m., 2 p.m. and 8 p.m. at Gizeh (Cairo), for the years 1902–10, Assiut 1900–1910 and Assuan 1901–1910.

Mean temperature Centigrade.

	Jan.	Feb.	March	April	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.
Gizeh near Cairo	10·8	12·3	14·7	19·1	23·1	25·4	26·7	26·5	24·4	21·7	17·0	12·6
Assiut	11·2	13·1	16·6	21·8	26·2	28·8	29·3	29·0	26·2	23·4	17·7	13·7
Assuan	14·5	17·1	20·3	25·4	29·5	32·0	32·6	32·2	30·3	27·4	21·2	16·3

Relative humidity (Saturation 100).

	80	72	68	60	56	57	62	67	71	75	78	82
Gizeh near Cairo												
Assiut	69	63	55	43	37	36	40	45	57	62	66	66
Assuan	52	45	40	34	36	34	30	30	35	38	45	49

Determination of the Species.

In the case of small collections every individual flea in the tube was separately examined under a compound microscope, but of the large collections, comprising from 2000 to 10,000 fleas in a single tube, a sample only was examined. These samples consisted of at least 500, and in some instances of 1000 to 2000 specimens, each of which was identified. The number of specimens noted in the Tables as examined must not be taken as affording any guide to the relative numbers captured on the various individual hosts mentioned, nor as evidence of a seasonal or geographical prevalence. The statistics relating to

¹ In tabulating our results we have followed on the lines laid down by Chick and Martin (1911), the present paper forming an addendum to their work and filling the gap caused by the fact that there were no figures relating to Egyptian fleas available at the time they wrote.

this side of the research are in course of arrangement and will be published later.

We take this opportunity of recording our thanks to Dr Harriette Chick for assistance with the earlier consignments, and to the Hon. N. Charles Rothschild for his kindness in checking some of our determinations of species, especially in regard to *Xenopsylla chephrenis* and *X. cleopatrae*.

Notes on the Species represented in the Collections.

Xenopsylla cheopis, as was to be expected, makes up by far the greatest bulk of the collections as a whole. When the animals from which the fleas were taken were trapped on land the percentage of this species normally present varies from 90 % to 100 %.

The few exceptions to this general rule are afforded by such hosts as *Gerbillus pyramidum*, a specimen caught wild, yielding a high percentage of *Xenopsylla cleopatrae*, a species of hedgehog which showed a distinct preponderance of *Ctenocephalus felis*, a weasel carrying *Echidnophaga gallinaceus* and *Acomys cahirinus* from Cairo, which had a marked infestation of *Xenopsylla chephrenis*. On hosts trapped in the Nile boats the relative numbers of *X. cheopis* declined, their place being occupied by *Leptopsylla musculi*.

Leptopsylla musculi. It is remarkable how dependent the occurrence of this species in the collection is upon the trapping of rodents from the Nile boats (feluccas) during the cooler season of the year.

The percentage captured upon animals in dwellings is infinitesimal, whereas in the collections from feluccas it sometimes exceeds that of *X. cheopis*, rising in one instance to 85 %.

It is no easy matter to disentangle and assess correctly the various factors which may contribute to this result. As already pointed out in our remarks on localities, *M. norvegicus* is the dominant rodent on the Nile boats.

Further, from a considerable mass of data relating to the rat and rat flea infestation of Lower Egypt collected by Professor Bitter¹ and Dr Charles Todd of the Hygienic Institute in Cairo, it would appear that *M. norvegicus* occurs much more commonly as a house-rat in Lower Egypt than in Upper Egypt, and also that *L. musculi* is a much more

¹ We desire to acknowledge the courtesy of Professor Bitter and Dr Charles Todd in permitting us the privilege of becoming acquainted with their observations.

TABLE II.

Showing species of Fleas found on various Hosts trapped in Houses.

Locality			Date of collection	Species of Animal	Number of specimens examined	Percentage of each species of Flea							
Town or Village	District (Markaz)	Province (Mudiriya)				<i>Pulex irritans</i>	<i>Echidnophaga gallinacea</i>	<i>Xenopsylla cheopis</i>	<i>Xenopsylla cheppensis</i>	<i>Xenopsylla cinerascens</i>	<i>Ceratophyllus fuscatus</i>	<i>Ceratophyllus fides</i>	<i>Lepopsylla muscardi</i>
Cairo			Nov. 1913	<i>Mus rattus</i>	208	—	—	91.8	8.1	—	—	0.4	—
			Nov. 1913	<i>Acomys cahirinus</i>	212	—	—	3.3	96.2	—	—	17.7	—
			May-Aug. 1913	Weasel	45	82.2	—	—	—	—	—	—	—
Kafr-el-Mansura	Minia		Dec. 1911	<i>Mus rattus</i>	114	—	0.8	99.1	—	—	—	—	—
			Sept. 1913	<i>Mus rattus</i>	540	—	—	100	—	—	—	—	
				<i>Mus norvegicus</i>	89	—	5.6	94.3	—	—	0.2	—	
Abnoub	Abnoub	Assiut	Jan. 1912	<i>Mus rattus</i>	1058	0.7	—	98.9	—	—	—	—	
				<i>Mus norvegicus</i>	1	—	—	1 flea only	—	—	—	—	
				Mouse	1	1 flea only	—	—	—	—	—	—	
Assiut	Assiut	Assiut	Dec. 1911 to Jan. 1913	<i>Mus rattus</i>	13,131	0.35	—	98.95	—	—	0.68	0.02	
				<i>Mus norvegicus</i>	2664	0.37	—	97.94	—	—	1.50	0.19	
				<i>Acomys cahirinus</i>	5839	0.47	—	98.25	0.74	—	0.52	0.02	
El Filia	Aboutig	Assiut	July, Aug. 1913	<i>Arviculhis niloticus</i>	2060	0.09	—	98.8	—	—	—	0.04	—
				<i>Crocidura olivieri</i>	5	—	—	(5)	—	—	—	—	
				<i>Mus rattus</i>	623	—	—	100	—	—	—	—	
Edfa and Kelfaw	Sohag	Girga	July 1912	<i>Acomys cahirinus</i>	196	—	—	100	—	—	—	—	
				<i>Mus rattus</i>	185	—	—	100	—	—	—	—	
				<i>Acomys cahirinus</i>	125	—	—	100	—	—	—	—	
Kous and neighbouring villages	Kous	Kena	April to June 1912	<i>Arviculhis niloticus</i>	32	—	—	96.8	—	—	3.2	—	
				<i>Crocidura olivieri</i>	11	—	—	100	—	—	—	—	
				<i>Mus rattus</i>	845	0.1	—	99.8	—	—	—	—	
				<i>Mus norvegicus</i>	325	—	—	100	—	—	—	—	
				<i>Acomys cahirinus</i>	424	0.9	—	99	—	—	—	—	
Konombo	Assuan	Assuan	Nov. 1911 to Feb. 1912	<i>Arviculhis niloticus</i>	738	0.4	—	99.5	—	—	—	—	
				<i>Mus norvegicus</i>	932	—	—	99.9	—	—	—	—	
				<i>Arviculhis niloticus</i>	1754	—	—	99.9	—	—	—	—	
				<i>Gerbillus pyramidalis</i>	14	—	—	100	—	—	—	—	
					12	8	—	91.6	—	—	—	—	

The number in brackets records the actual specimens and is not a percentage.

TABLE III.

Showing the number and species of Fleas taken from Rodents trapped on Feluccas (Nile boats) at Abnoub, Assiut and Komombo.

Locality			Percentage or number of each species of Flea										
Town or village	District (Markazi)	Province (Mudiriya)	Date of Collection	Species of Animal	Number of specimens examined	<i>Pulex irritans</i>	<i>Echidnophaga gallinacea</i>	<i>Xenopsylla cheopis</i>	<i>Xenopsylla chephrenis</i>	<i>Xenopsylla cleopatrae</i>	<i>Ceratophyllus fuscatus</i>	<i>Cleophtatus fella</i>	<i>Leplopsylla muscui</i>
Abnoub	Assiut	Assiut	Jan. 1912	<i>Mus norvegicus</i>	32	3.1	—	56.2	—	—	—	6.2	34.3
				<i>and rattus</i>		(1)	—	(1)	—	—	(3)	—	
Assiut	Assiut	Assiut	Feb. 1912 to Jan. 1913	<i>Acomys cahirinus</i>	5	0.8	—	69.2	—	—	—	0.1	29.9
				<i>Mus rattus</i>		0.1	—	49.3	0.03	—	0.06	0.1	50.1
Konombo	Assiut	Assiut	Nov. 1911	<i>Mus norvegicus</i>	3261	3.7	0.1	78.4	—	—	—	—	17.7
				<i>Acomys cahirinus</i>	79	—	—	97.9	—	—	—	0.7	—
				<i>Mus rattus</i>	143	—	1.4	98.7	—	—	—	1.2	—
				<i>Mus norvegicus</i>	478	—	—	—	—	—	—	—	—

Numbers in brackets record the actual specimens, not percentages.

TABLE IV.

Showing that *Leptopsylla musculi* is relatively more numerous than *Xenopsylla cheopis* during the cooler months of the year.

Fleas taken on Rodents trapped on Feluccas (Nile boats) at Assiut.

Date of Collection		Percentage of each species of Flea									
Species of Animal	Number of specimens examined	<i>Pulex irritans</i>	<i>Echidnophaga gallinacea</i>	<i>Xenopsylla cheopis</i>	<i>Xenopsylla cheoprensis</i>	<i>Ceratophyllus fuscatus</i>	<i>Clenoccephalus fella</i>	<i>Leptopsylla musculi</i>			
<i>Mus rattus</i>	319	1.5	—	48.5	—	—	0.3	49.5			
	206	—	—	99.5	—	—	—	0.4			
	60	—	—	73.3	—	—	—	26.6			
<i>Mus norvegicus</i>	1458	0.1	0.06	13.4	0.06	0.06	0.2	85.9			
	536	0.1	0.1	74.8	—	0.1	—	24.6			
	262	0.3	0.3	99.2	—	—	—	—			
	902	—	0.1	79.7	—	—	0.1	20.0			
	103	—	—	73.3	—	—	—	26.6			
<i>Acomys cahirinus</i>	32	(1)	—	(18)	—	—	—	(13)			
	34	(2)	—	(32)	—	—	—	—			
	3	—	—	(3)	—	—	—	—			
	10	—	—	(9)	—	—	—	(1)			

Numbers in brackets record actual specimens, not percentages.

TABLE V.

Showing species of Fleas obtained from a variety of sources not included in the previous Tables.

Locality			Date of Collection	Host or Source from which Fleas were obtained	Number of specimens examined	Percentage or number of each species of Flea							
Town or Village	District (Markaz)	Province (Mudiriya)				<i>Psylla irritans</i>	<i>Echidnopsylla hirsuta</i>	<i>Xenopsylla cheopis</i>	<i>Xenopsylla cheopis</i>	<i>Xenopsylla cleopatrae</i>	<i>Ceratophyllus fasciatus</i>	<i>Ceratophyllus frits</i>	<i>Leptopsylla musculi</i>
Assiut	Assiut	Assiut	May 1913	Dog	284	4.9	—	—	—	—	—	95.1	—
Assiut	Assiut	Assiut		Human host on clothing	538	100	—	—	—	—	—	—	—
Abnoub	Abnoub	Assiut	Jan. 1912	<i>Mus rattus</i> and mouse	4	—	—	(4)	—	—	—	—	—
Abnoub	Abnoub	Assiut	Jan. 1912	<i>Mus rattus</i> and <i>norvegicus</i>	431	0.6	—	93.7	—	—	—	—	5.5
Abnoub	Abnoub	Assiut	Jan. 1912	Clothes of plague patient	44	(43)	—	(1)	—	—	—	(17)	—
El Motiaa	Assiut	Assiut	May 1912	Hedgehog	18	(1)	—	—	—	—	—	—	—
Kous	Kous	Kona	May 1912	Clothes of plague patient	1	(1)	—	—	—	—	—	—	—
Komombo	Assiut	Assiut	Nov. 1911	<i>Acomys</i> from Nile bank	14	—	—	(14)	—	—	—	—	—
Komombo	Assiut	Assiut	Feb. 1912	<i>Gerbillus pyramidalis</i>	20	—	—	(2)	—	(18)	—	—	—
Komombo	Assiut	Assiut	Feb. 1912	Rats' nests in houses	121	—	—	100	—	—	—	—	—
Komombo	Assiut	Assiut	Feb., Mar. 1912	Plague infected houses	2821	—	—	100	—	—	—	—	—
Komombo	Assiut	Assiut	Feb. 1912	Clothes of rat catchers in infected houses	193	4	—	96	—	—	—	—	—
Komombo	Assiut	Assiut	Feb. 1912	Clothes of plague patients	15	(2)	—	(13)	—	—	—	—	—

Numbers in brackets record actual specimens, not percentages.

prevalent species of flea on house-rats in Lower Egypt than in Upper Egypt.

From these considerations it might be inferred that the occurrence of *L. musculi* and of *M. norvegicus* are inter-related, but on the other hand this view is not supported by any evidence that *L. musculi* favours *M. norvegicus* rather than *M. rattus*, whether the animals are trapped in feluccas in Upper Egypt or in the native dwellings of Lower Egypt.

The two most plausible solutions are either that *L. musculi* is a species mainly restricted to Lower Egypt and that the feluccas are infested from this source or that the conditions on the Nile boats are more favourable for it than those in the rat-nests on land in Upper Egypt.

It seems definite, however, that the land conditions in Upper Egypt are unfavourable to the continued existence of *L. musculi*, though whether because of a small mouse population, or owing to the climatic conditions or for some other reason, is not clear.

Ceratophyllus is represented in these collections by three specimens of *fasciatus*, all taken from *M. norvegicus* trapped on feluccas during the first half of the year. From these circumstances and the fact that the species is present in small numbers on rats captured in Lower Egypt, we again get a suggestion of importation from the North, but curiously it is *M. rattus* which carries the larger number of specimens according to the unpublished data we have been privileged to see.

Xenopsylla cleopatrae was found only on one occasion, the host being a *Gerbillus pyramidum* captured in February, 1912, at Komombo. This flea would appear to be in no way associated with the usual rodent population of inhabited districts.

Xenopsylla chephrenis. Of the 265 specimens of this flea found in these collections, by far the larger number (221) are from Cairo; the remainder (44 specimens) were captured at Assiut. Of these latter 43 were on *Acomys* trapped in houses and one from a *M. norvegicus*, caught on a Nile boat at that town. The distribution on the hosts of the species trapped at Cairo is also significant; 204 of the fleas being found on *Acomys*, the remaining 17 on *M. rattus*. See Table II.

Ctenocephalus felis. Of the genus *Ctenocephalus*, *Ct. felis* alone is present in these collections, and although the percentage is low in comparison with that of *Xenopsylla cheopis* or *Leptopsylla musculi*, the actual number of specimens is large enough to justify the expectation of finding *Ct. canis* also, if this species occurs in Egypt. Probably,

as in other tropical countries, *Ct. felis* does duty for both feline and canine animals in Upper Egypt.

Echidnophaga gallinacea is rare considering the probable chances of intercourse between rats and fowls.

Pulex irritans. In view of the close association obtaining between rats and man under the conditions in which a large section of the human population of Egypt live, the percentage of this species found on trapped rats must be considered as extremely small, especially as human infestation with *Pulex irritans* is sometimes very heavy (Table V).

SEX PROPORTIONS. The very marked discrepancy between the number of male and female specimens of *Xenopsylla chephrenis* caused us to keep a record of the sexes of other species in the tubes that had not previously been examined when this discrepancy was first noted. The results set forth in the following table show that some difference in the proportion of the sexes is also present in *X. cheopis*, although to a much slighter extent, the difference only amounting to 7 %.

TABLE VI.

Proportion of sexes in fleas captured on Rats and other Rodents, and on Man.

Species	Number examined	Males	Females	Excess
<i>Xenopsylla cheopis</i>	8498	3948 (46.5 %)	4550 (53.5 %)	602 (7 %)
<i>Xenopsylla chephrenis</i>	259	36 (13.9 %)	223 (86.1 %)	187 (72.2 %)
<i>Pulex irritans</i>	547	329 (60.1 %)	218 (39.9 %)	111 (20.2 %)

As the numbers dealt with in arriving at this conclusion are considerable, over 8000 specimens from 18 parcels collected either at different seasons or in separate localities, it is probably justifiable to suppose that this want of balance is general and implies some variation in the habits of the sexes. With regard to *X. chephrenis*, although the number of specimens is not large, the discrepancy is so striking that there seems no doubt that some deep-seated divergence in the habit of the sexes is indicated. The explanation we would suggest is that the males wait in the nest to fertilize the females as they emerge from their cocoons, while the instinct of the female is to attach herself more closely to the host and feed as continuously as possible, as by this procedure egg development will be more rapid, and the chances of founding new colonies when the young of the host scatter will be enhanced.

In the terms of such a theory the position of *X. cheopis* is either that of a species in which the ancestral habit (possibly originating in a desert habitat) is waning with its adaptation to semi-domesticated hosts, or, which seems a less plausible proposition, that *X. chephrenis* is developing to excess the ancestral habits of the group which are retained by *X. cheopis*.

In the case of *P. irritans* it is doubtful as to how far the excess of males is a chance phenomenon, as the numbers, which are not large, relate to two parcels only.

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- CHICK, H., and MARTIN, C. J. (1911). The fleas common on rats in different parts of the world and the readiness with which they bite man. *Journ. of Hygiene*, xi. 122.

ANALYSIS OF THE EFFECT OF NUCLEIN AND NUCLEIC ACID ON THE NORMAL ANTIBODY.¹

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PART I.

THE fact was observed some years ago by various experimenters—Mikulicz (1904¹), Miyake (1904²), Renner (1905³)—that an increase in the natural immunity to infection frequently followed the injection of substances of the class of nucleins or nucleo-proteins derived from tissues or bacterial cells. The analysis of this effect on natural immunity has not yet been adequately investigated. It was thought at first that this enhanced resistance was due to the marked leucocytosis produced by these substances, but more recent work has shown that such an explanation can only be considered as incomplete, in view of the demonstration of actual changes in the amount and character of the normal antibodies of the serum.

Thus Hahn (1897⁴) showed that injections of a yeast nuclein into dogs produced a rise in the bactericidal power of the blood for *B. coli*, *B. pyocaneus*, and *Staphylococcus pyogenes aureus*. This rise was most marked five hours after the injection, but was of a fleeting character, and coincided with the stage of hyperleucocytosis. Huggard and Morland (1905⁵) employing yeast in the treatment of tuberculosis (20 grms. daily), noted a rise in the opsonic index to the tubercle bacillus. They found that this rise, which was in no case very marked, an increase of 0.62 in the index being the greatest obtained, followed a leucocytosis, which set in soon after administering the yeast, lasting three to four hours. Ledingham and Bulloch (1906⁶) went into the question of the effect of leucocytosis-producing substances on the opsonic index, and concluded that nuclein alone, out of a number of preparations employed, brought about a rise in the opsonic index.

At the suggestion of Dr. Ledingham, an investigation of this action of the nucleins and nucleic acid was undertaken by me, with a

¹ [Received January 16, 1914.] Communicated to the Pathological Society of Great Britain and Ireland, January 9-10, 1914 (see this Journal, vol. xviii. p. 416).

view to determining what effect, if any, these substances have on the normally occurring immune bodies, and with the hope that at the same time by working with the simplest forms of antigen some light might be thrown on antibody response in general.

Technique of Experiments.

The samples of blood were allowed to coagulate at room temperature for thirty minutes, centrifuged, and the serum pipetted off immediately. The sera were kept in the cold room until the opsonic determination was made, which was generally done the same day on which the samples were taken.

Washed human leucocytes (S.P.B.) were employed throughout, and an emulsion of a sixteen hours' growth of *Staphylococcus pyogenes aureus* at 37° C. was used as the test organism.

Dilutions of the serum were employed in most cases. The mixture of equal parts of serum, washed leucocytes, and organisms was placed in the incubator at 37° C. for fifteen minutes, being kept in a shaker during this period. In every case a count of 100 to 200 leucocytes was made.

EFFECT OF LEUCOCYTOSIS-PRODUCING SUBSTANCES ON THE OPSONIC INDEX.

The first experiments performed confirmed the work of Ledingham and Bulloch, it being found that a yeast nuclein (Parke Davis, Sol. No. 1, containing 5 per cent. nucleic acid) injected in doses of 3 c.c. intravenously, produced a marked rise in the phagocytic index, whereas similar injections of tallianine and sodium cinnamate preparations, which both produce a marked leucocytosis, were without effect on the opsonin content of the serum (see Experiments I., II., and III.).

Experiment I.

RABBIT—Intravenous Injection of 3 c.c. Nuclein (Parke Davis).				RABBIT (Control).		
	Dilutions of Serum.			Dilutions of Serum.		
	1/1	1/5	1/10	1/1	1/5	1/10
Before injection	2.68	4.03	0.58	2.60	3.75	1.12
Four hours after injection .	5.79	1.13	0.33	2.36	4.15	0.90

It will be noticed that there is a marked rise in the phagocytic coefficient of the injected rabbit four hours after the injection, which represents a rise in the opsonic index from 1.03 to 2.45.

Experiment II.

RABBIT—Intravenous Injection of 3 c.c. Sodium Cinnamate (5 per cent. solution).				RABBIT (Control).		
	Dilutions of Serum.			Dilutions of Serum.		
	1/1	1/5	1/10	1/1	1/5	1/10
Before injection	6.08	4.05	0.84	2.92	3.58	0.50
Four hours after injection .	5.60	3.60	0.43	2.24	2.44	0.57

Experiment III.

RABBIT—Intravenous Injection of 3 c.c. Tallianine.				RABBIT (Control).		
	Dilutions of Serum.			Dilutions of Serum.		
	1/1	1/5	1/10	1/1	1/5	1/10
Before injection	3.95	6.72	3.21	3.73	4.16	3.65
Four hours after injection .	3.76	6.23	3.45	4.03	4.53	3.85

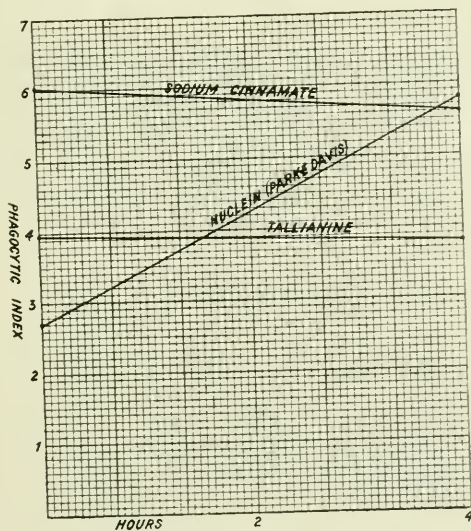


FIG. 1.

In neither case is there any alteration in the opsonic value of the sera after the injection. These results are seen plotted as curves in Fig. 1. In the case of Experiment I, it will be seen that though the

serum after injection has a phagocytic index more than double that of the serum before injection, on diluting with normal saline it rapidly falls below the latter. This is to some extent what Marshall (1908⁷) showed, namely, that after injection of an antigen, the opsonic value of a serum falls much more rapidly than in the case of a normal serum, though in this case the phenomenon is much more marked. This effect will be seen to occur consistently throughout these experiments.

TIME OF ONSET OF RISE.

The next point determined was the exact time, after the injection, at which this rise commenced. In the first experiment samples of blood were taken 1 hour, 2 hours, $2\frac{1}{2}$ hours, 3 hours, and $3\frac{1}{2}$ hours after the injection, with the following result:—

Experiment IV.

RABBIT—Intravenous Injection of 3 c.c. Nuclein (Parke Davis, Solution No. 1).					
Before Injection.	1 hour after.	2 hours after.	$2\frac{1}{2}$ hours after.	3 hours after.	$3\frac{1}{2}$ hours after.
5.30	9.30	12.79	10.67	8.57	11.17

It will be seen from this that even one hour after injection the phagocytic index of the serum has risen quite markedly, and remains up throughout the different readings, although fluctuating a little.

In the next experiment the samples of blood were taken at more frequent intervals, and the following figures obtained:—

Experiment V.

RABBIT—Intravenous Injection of 3 c.c. Nuclein (Parke Davis, Solution No. 1).					
Before Injection.	$\frac{1}{4}$ hour after.	$\frac{1}{2}$ hour after.	1 hour after.	2 hours after.	4 hours after.
3.63	3.35	5.6	4.89	4.47	6.66

Here the first indication of the rise occurs thirty minutes after the injection.

The third experiment gave much the same result.

Experiment VI.

RABBIT—Intravenous Injection of 3 c.c. Nuclein (Parke Davis).		RABBIT (Control).
Before injection . . .	5.60	5.66
Twenty minutes after . .	10.13	5.76
Thirty minutes after . .	12.05	...

The first indication here is twenty minutes after the injection. The last experiment on this point was done with a view to comparing the opsonic values of the serum before, and half an hour after, the injection, by the method of dilutions.

Experiment VII.

RABBIT—Intravenous Injection of 3 c.c. Nuclein (Parke Davis, Solution No. 1).		
Dilutions of Serum.	Before Injection.	30 Minutes after Injection.
1/1	4.70	12.33
1/5	0.90	3.05
1/10	0.15	0.26
1/20	...	...
1/40	...	...
1/80	...	...

Despite the fact that in the undiluted state the phagocytic index of the serum thirty minutes after injection is almost three times that of the serum before injection, the two had fallen to practically the same value on reaching a dilution of $\frac{1}{20}$ th. This dilution effect has already been referred to in the case of Experiment I., and we see here that the method of Klein does not give us a true estimate of the opsonic value of the serum in this case. The results of these experiments are represented graphically in Fig. 2.

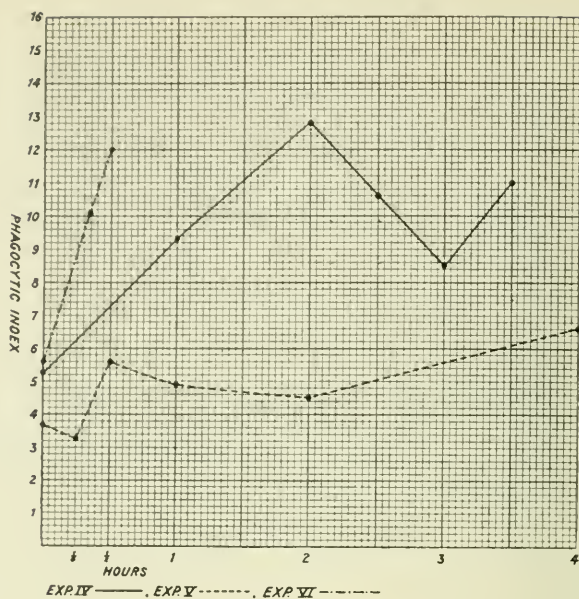


FIG. 2.

DURATION OF THE RISE.

The next experiments were performed to ascertain the extent and duration of this increase in the opsonic power of the serum. Samples of blood were taken before injection, and 1 hour, 4 hours, $7\frac{1}{2}$ hours, and 24 hours after.

Experiment VIII.

RABBIT—Intravenous Injection of 3 c.c. Nuclein (Parke Davis).					RABBIT (Control).		
	Dilutions of Serum.				Dilutions of Serum.		
	1/1	1/5	1/10	1/20	1/1	1/5	1/10
Before injection	5.73	4.25	1.61	...	4.45	4.12	1.79
1 hour after injection	7.41	5.19	1.82	...	..	...	...
4 hours	5.37	5.40	1.73	1.10	...	...	...
$7\frac{1}{2}$ "	5.18	3.60	1.13	0.36	...	...	...
24 "	...	5.97	2.12	...	3.28	6.22	3.28

No conclusion can be drawn from this experiment, the rabbit evidently having reacted only very slightly to the injection, if at all. In the next experiment an enormous rise was obtained.

Experiment IX.

RABBIT—Intravenous Injection of 3 c.c. Nuclein. (Parke Davis).					RABBIT (Control).		
	Dilutions of Serum.				Dilutions of Serum.		
	1/1	1/5	1/10	1/20	1/1	1/5	1/10
Before injection . . .	4.78	2.87	0.53	...	7.08	6.01	1.74
1 hour after injection	23.06	...	1.44	0.41	...	...	...
4 hours ,, ,,	18.80	...	1.16	0.22	...	...	...
7½ ,, ,, ,,	15.87	...	0.62	...	5.72	3.81	1.49
24 ,, ,, ,,	4.18	...	1.39	0.70	...	...	...

This marked rise already present 1 hour after injection is still in evidence 7½ hours after, but has completely disappeared in 24 hours. The effect of dilution is again well brought out, for, measured by this method, the different samples of sera differ very little from one another, whilst in the undiluted state their opsonic values are widely divergent. Finally, the pooled sera of three injected rabbits were examined, being controlled by the pooled sera from three normal rabbits, and the following figures obtained:—

Experiment X.

Pooled Sera from Three RABBITS. Each received 2 c.c. Nuclein (Parke Davis) Intravenously.					Pooled Serum from Three RABBITS (Controls).		
	Dilutions of Serum.				Dilutions of Serum.		
	1/1	1/5	1/10		1/1	1/5	1/10
Before Injection . . .	4.46	2.95	0.84		4.56	7.94	...
1 hour after injection . . .	11.25	1.96	0.45		...	...	...
5 hours ,, ,, ,,	6.68	1.34	0.76		4.27	8.64	2.06
24 ,, ,, ,, ,,	5.02	3.55	1.18		4.60	8.31	1.96

Here again a marked rise results from the injection, although it falls again more rapidly than it did in the previous experiment. The results of Experiments VIII., IX., and X. are seen plotted as curves in

Fig 3. This increase, then, in the opsonic index, brought about by the injection of nuclein, is first observed 20 to 30 minutes after the intravenous injection, and is still marked $7\frac{1}{2}$ hours later, but has completely disappeared in 24 hours.

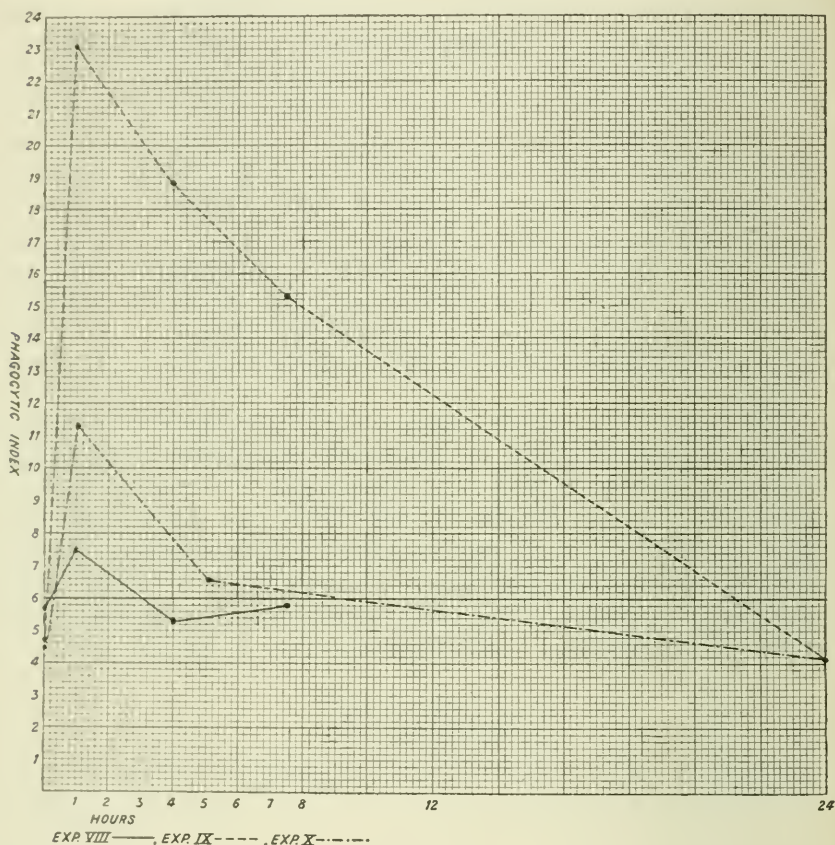


FIG. 3.

THE THERMO RESISTANCE OF THE SERUM OF ANIMALS INJECTED WITH NUCLEIN.

With a view to determining what effect heat had on the opsonin of the serum of the injected rabbit, as compared with the effect it had on the normal rabbit serum, the following experiment was performed. Samples of blood were taken from two rabbits, one of which was then given an intravenous injection of 3 c.c. nuclein (Parke Davis). The sera resulting from the second samples of blood taken four hours later were divided into two parts, one of which was heated for 30 minutes at 56° C. The phagocytic indices of the various sera were then determined, and the following result obtained:—

Experiment XI.

Dilutions of Serum.	RABBIT—Intravenous Injection of 3 c.c. Nuclein (Parke Davis).				RABBIT (Control).	
	Before Injection.	4 hours after Injection.		Before Injection.	4 hours later.	
		Serum unheated.	Serum inactivated.		Serum unheated.	Serum inactivated.
1/1	2.68	5.79	0.44	2.60	2.36	0.84
1/5	4.03	1.13	0.14	3.75	4.15	0.22
1/10	0.58	0.33	0.07	1.12	0.90	0.18

It will be seen that the phagocytic index of the serum from the injected rabbit falls more rapidly than that of the normal serum on being heated for thirty minutes at 56° C. It would appear then, from this experiment that there is no increase in the thermostable opsonin normally present in the serum. Very similar results were obtained in the two following experiments, performed in the same manner as the one just described.

Experiment XII.

Dilutions of Serum.	RABBIT—Intravenous Injection of 3 c.c. Nuclein (Parke Davis).				RABBIT (Control).	
	Before Injection.	4 hours after Injection.		Before Injection.	4 hours later.	
		Serum unheated.	Serum inactivated.		Serum unheated.	Serum inactivated.
1/1	3.97	6.86	0.38	3.07	2.55	0.40
1/5	5.76	0.82	0.23	6.08	6.11	0.29
1/10	0.96	0.28	0.14	1.55	1.35	0.19

Experiment XIII.

Dilutions of Serum.	RABBIT—Intravenous Injection of 3 c.c. Nuclein (Parke Davis).				RABBIT (Control).	
	Before Injection.	4 hours after Injection.		Before Injection.	4 hours later.	
		Serum unheated.	Serum inactivated.		Serum unheated.	Serum inactivated.
1/1	8.42	27.68	0.33	6.75	7.14	1.12
1/5	14.89	2.97	0.19	20.18	12.34	0.38
1/10	3.57	1.75	0.14	6.63	6.04	0.41

EFFECT OF DIFFERENT PREPARATIONS OF NUCLEIC ACID.

Several other preparations were then tested with regard to their power to raise the opsonic index on intravenous injection. The first to be employed was a sodium nucleinate (Merck), prepared from yeast. A 5 per cent solution in normal saline was made up, and sterilised by autoclaving for 20 minutes at 120° C. (see Experiment XIV.).

Experiment XIV.

RABBIT—Intravenous Injection of 3 c.c. 5 per cent. Sodium Nucleinate (Merck).				RABBIT (Control).		
	Dilutions of Serum.			Dilutions of Serum.		
	1/1	1/5	1/10	1/1	1/5	1/10
Before injection .	3.49	1.00	0.23	7.57	1.88	0.80
4 hours after injection	7.48	0.88	0.48	8.11	1.27	0.47

A marked rise is seen to have occurred in four hours, the opsonic index having risen from 0.46 to 0.92. The duration of the rise occasioned by this preparation was next determined (see Experiment XV.).

Experiment XV.

RABBIT—Intravenous Injection of 3 c.c. 5 per cent. solution of Sodium Nucleinate (Merck).				RABBIT (Control).		
	Dilutions of Serum.			Dilutions of Serum.		
	1/1	1/5	1/10	1/1	1/5	1/10
Before injection .	5.00	4.39	2.81	3.39	8.44	2.67
1 hour after injection	10.40	1.62	0.49	...	...	...
4 hours after injection	9.46	2.29	0.45	...	...	...
7 hours after injection	11.38	2.08	0.75	3.75	6.92	2.37
24 hours after injection	4.59	2.29	1.10	3.22	9.80	3.07

We get here a similar result to that obtained in the case of the Parke Davis preparation. The rise, though not quite so great, is still marked 7 hours after the injection, but has disappeared entirely in 24 hours.

The next preparation employed was a yeast nucleic acid (Boehringer). A solution of this was prepared by dissolving up 1 grm. of the powder in N/2 soda, further soda being added until the solution was neutral to phenolphthalein. The solution was then made up to 20 c.c. with distilled water, and 1.5 grms. NaCl added to make the solution isotonic. This solution was sterilised by heating to 60° C. for one hour on four successive days, being incubated at 37° C. between each heating. This solution was injected intravenously in doses of 3 c.c. (see Experiment XVI.).

Experiment XVI.

RABBIT—Intravenous Injection of 3 c.c. 5 per cent. Sodium Nucleinate solution (Boehringer).				RABBIT (Control).		
	Dilutions of Serum.			Dilutions of Serum.		
	1/1	1/10	1/20	1/1	1/10	1/20
Before injection .	2.20	1.91	0.58	3.09	3.36	1.02
4 hours after in- jection	3.82	0.90	0.52	2.36	2.42	0.92

Practically no rise has occurred here. This experiment was repeated, observations being taken over a longer period (see Experiment XVII.).

Experiment XVII.

RABBIT—Intravenous Injection of 3 c.c. 5 per cent. solution of Sodium Nucleinate (Boehringer).				RABBIT (Control).		
	Dilutions of Serum.			Dilutions of Serum.		
	1/1	1/10	1/20	1/1	1/10	1/20
Before injection .	3.82	8.57	4.88	4.16	1.20	0.59
1 hour after in- jection	3.78	12.41	5.60	...	...	...
4 hours after in- jection	3.54	9.49	5.63	3.36	1.95	0.95
7 hours after in- jection	2.05	6.49	2.83	...	...	...
24 hours after in- jection	2.07	5.04	1.92	4.05	1.42	0.69

It is seen, however, that here again no rise in the phagocytic index has taken place, in fact it has fallen to a certain extent. This preparation was tried again, but on this occasion a suspension of the nucleic acid in normal saline was injected instead of the sodium salt. This suspension contained 5 per cent. nucleic acid, a small portion of which had gone into solution. It was acid to phenolphthalein and litmus, and was injected without previous sterilisation. The result was as follows:—

Experiment XVIII.

RABBIT—Intravenous Injection of 3 c.c. Suspension of Nucleic Acid (Boehringer).				RABBIT (Control).		
	Dilutions of Serum.			Dilutions of Serum.		
	1/1	1/5	1/10	1/1	1/5	1/10
Before injection .	3.62	5.67	0.96	2.77	2.53	0.58
4 hours after injection	2.93	4.90	0.87	2.75	2.59	0.54

Again no rise has occurred. It may be concluded, then, from these experiments that this preparation of nucleic acid is without effect on the opsonin of the serum. However, this preparation was found to be a pure one as judged by its phosphorus and nitrogen content, the figures being P. 8.8 per cent., N. 14.3 per cent.; those given by Levene for a yeast nucleic acid being P. 8.69 per cent., N. 14.8 per cent. This being the case, it seems probable that the effect on the opsonin which has been observed in the case of the first two preparations is not due to the nucleic acid radicle, but to the presence of some impurity.

This is in concordance with what has been found with regard to the antigenic properties of nuclein and nucleic acid. Thus Taylor (1908⁸) was unable to obtain a cytolytic immune body by injecting rabbits with nucleic acid prepared from salmon sperm, and Gideon Wells (1911⁹) failed to sensitise guinea-pigs with a sodium nucleinate prepared from cod sperm, a second injection of either the same nucleinate or the albumin from cod sperm producing no anaphylactic symptoms. More recently Abderhalden and Kashiwado (1912¹⁰) were unable to produce anaphylaxis in animals with either nucleic acid from thymus or a yeast nucleic acid. Pearce and Jackson (1906¹¹), working with animal "nucleo-proteins," though finding these substances antigenic (cytotoxines), were unable to demonstrate any organ specificity. In a review of the work done on the nucleo-proteins as antigens (Gideon Wells, 1913¹²), Wells states that his experience with materials of this class is precisely that of Pearce and Jackson. The earlier precipitates

obtained by acidifying the watery extract of a tissue, the so-called "nucleo-protein," containing nuclein and proteins, whether bound to one another or not one does not know, are unquestionably antigenic. The antibodies to which they give rise, however, are not definitely specific either for the nucleo-protein in question or for the organ from which they were derived. As the purification is repeated, more and more protein being removed, and approaching more and more to the pure nucleins, the preparations lose their power to produce anaphylactic shock. This loss in antigenic power, he considers, is to be expected, since neither components of nucleins—histone and protamine (Gay and Robertson, 1912, 1913¹³), and nucleic acid—possess antigenic properties. He concludes, therefore, that those preparations of nuclein and nucleic acid which act as antigens, are able to do so owing to the presence of some protein impurity, obtained in the first instance from the tissue or bacterial cells during the extraction of the nucleo-proteins, which are only removed with difficulty by repeated purification. It is possible that the same is true with regard to the power of nucleic acid to increase the opsonic power of the serum, and with this in view a pure tissue nucleic acid, kindly supplied by Dr. Levene, was examined. The nucleic acid was dissolved up in N/1 soda, the final solution being acid to phenolphthalein, and alkaline to tropeolin o o o.

The strength of the solution was 3 per cent., and it was divided into two portions, one of which was not sterilised, whilst the other was sterilised by filtration through a Berkefeld filter. On subsequent examination no organisms could be grown from the unfiltered solution. The effect of injection of these two solutions was then tested (see Experiment XIX.).

Experiment XIX.

	RABBIT — Intravenous Injection of 3 c.c. solution of Nucleic Acid (Levene), unfiltered.			RABBIT — Intravenous Injection of 3 c.c. solution of Nucleic Acid (Levene), filtered.			RABBIT (Control).		
	Dilutions of Serum.			Dilutions of Serum.			Dilutions of Serum.		
	1/1	1/5	1/10	1/1	1/5	1/10	1/1	1/5	1/10
Before injection	9·24	9·25	7·54	5·65	13·57	5·62	5·30	6·37	3·60
4 hours after injection	9·37	9·57	5·67	17·41	9·73	7·01	4·88	10·30	5·22

Here the unfiltered solution produces no rise at all, but, contrary to what might be expected, the filtered solution raised the phagocytic

index from 5.65 to 17.41. This experiment was repeated with the same result (see Experiment XX.).

Experiment XX.

	RABBIT — Intravenous Injection of 3 c.c. solution of Nucleic Acid (Levene), unfiltered.			RABBIT — Intravenous Injection of 3 c.c. solution of Nucleic Acid (Levene), filtered.			RABBIT (Control).		
	Dilutions of Serum.			Dilutions of Serum.			Dilutions of Serum.		
	1/1	1/5	1/10	1/1	1/5	1/10	1/1	1/5	1/10
Before injection	5.81	3.80	3.30	5.65	6.88	4.27	6.18	5.89	3.89
4 hours after injection	6.32	4.65	3.50	10.37	7.03	4.33	6.00	6.01	3.95

These results are plotted as curves in Fig. 4.

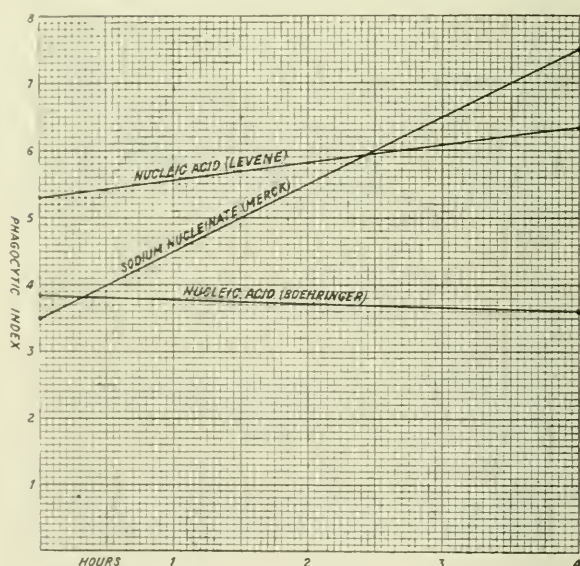


FIG. 4.

EFFECT OF CALCIUM SALTS ON THE OPSONIC INDEX.

The sodium salt prepared from this tissue nuclein (Levene), when injected without previous treatment, is without effect on the opsonic index of the serum; when, however, it is filtered through a Berkefeld candle it becomes active in this respect. Now it has been shown that

during the process of filtration through a Berkefeld filter a quite appreciable amount of calcium is added to the solution from the filter. It was possible in this case to demonstrate calcium in the filtered sodium nucleinate solution, whereas none could be found in the unfiltered solution. Similarly, if normal saline was passed through a Berkefeld filter, it could be shown to contain calcium, while before filtration it contained none (addition of ammonium oxalate to solution rendered alkaline with ammonia). Since it has been claimed (Ham-burger, 1910¹⁴) that calcium salts, even in very small traces, increase phagocytosis both *in vitro* and *in vivo*, it was thought that this might explain the rather unlooked-for results obtained in the last two experiments. To test this theory, normal saline was filtered through a Berkefeld and injected intravenously into a rabbit, the control animal receiving unfiltered normal saline. The filtered normal saline was found to contain calcium salts. The following result was obtained:—

Experiment XXI.

RABBIT—Intravenous Injection of 3 c.c. filtered Normal Saline.				RABBIT—Intravenous Injection of 3 c.c. Normal Saline.		
	Dilutions of Serum.			Dilutions of Serum.		
	1/1	1/5	1/10	1/1	1/5	1/10
Before injection .	5.12	6.44	2.88	2.89	8.91	5.06
4 hours after in- jection	5.82	7.82	2.88	2.97	7.70	2.85

The calcium salts present in the filtered normal saline have had no effect on the phagocytic index. It was thought that perhaps a solution of sodium nucleinate, being more viscous than the saline, might obtain more calcium in the process of filtration than the saline

Experiment XXII.

RABBIT—Intravenous Injection of 3 c.c. Sodium Nuclein (unfiltered), containing 5 per cent. CaCl_2 .				RABBIT—Intravenous Injection of 3 c.c. Normal Saline, contain- ing 0.5 per cent. CaCl_2 .		
	Dilutions of Serum.			Dilutions of Serum.		
	1/1	1/5	1/10	1/1	1/5	1/10
Before injection .	5.63	0.90	0.30	3.73	3.98	2.58
4 hours after in- jection	6.56	0.35	0.20	3.68	3.60	2.31

did, so 0.5 per cent. CaCl_2 was added to the unfiltered sodium nucleinate (Levene), already found to be without action, and it was then injected. The control rabbit received normal saline, to which had been added 0.5 per cent. CaCl_2 (see Experiment XXII.).

Here again very little change has resulted, but seeing that the rabbit which received the sodium nucleinate containing the calcium chloride was not full grown, and that it died $4\frac{1}{2}$ hours after the injection, one ought not, perhaps, to draw any conclusions from these results. Further experiments on this point are being made, but it would appear that the calcium is not the cause of this rise, and one will have to look elsewhere for the solution of this question.

EFFECT OF ALKALI ON THE OPSONIC INDEX.

Seeing that in the case of several of the preparations employed the solution injected was slightly alkaline ($[\text{H}^+]$ varying from 7.5 to 8.3), it was deemed necessary to test the effect of this amount of free alkali on the opsonic index. Thus N/1 soda was added to normal saline in sufficient quantity to bring the alkalinity up to $[\text{H}^+] = 9.3$, and 3 c.c. of this solution injected intravenously into a rabbit. The control received 3 c.c. normal saline.

Experiment XXIII.

RABBIT—Intravenous Injection of 3 c.c. Normal Saline rendered slightly Alkaline.				RABBIT—Intravenous Injection of 3 c.c. Normal Saline.		
	Dilutions of Serum.			Dilutions of Serum.		
	1/1	1/5	1/10	1/1	1/5	1/10
Before injection .	4.03	2.76	1.68	5.82	6.00	2.98
4 hours after injection.	2.49	2.44	0.96	5.33	7.77	2.57

The effect of the alkali here has been to lower the phagocytic index somewhat. This experiment was repeated with a similar result (see Experiment XXIV.).

It would appear then that alkali injected intravenously, if anything, slightly lowers the opsonic power of the serum.

THE EFFECT OF DILUTION ON THE SERUM OF ANIMALS INJECTED WITH NUCLEIN.

Perhaps one of the most interesting points brought out by these experiments is the rapidity with which the opsonic value of the serum, from the injected animal, falls on dilution, as compared with

Experiment XXIV.

RABBIT—Intravenous Injection of 3 c.c. Normal Saline (alkaline).

RABBIT—Intravenous Injection of 3 c.c. Normal Saline.

	Dilutions of Serum.			Dilutions of Serum.		
	1/1	1/5	1/10	1/1	1/5	1/10
Before injection .	4.79	2.47	0.53	2.69	3.90	1.75
4 hours after injection	3.54	2.37	0.84	...	4.00	1.70

the normal serum. This point has already been briefly referred to and is well brought out in Experiment XIII., the results of which are rendered graphically in Fig. 5. In the higher dilutions it is quite

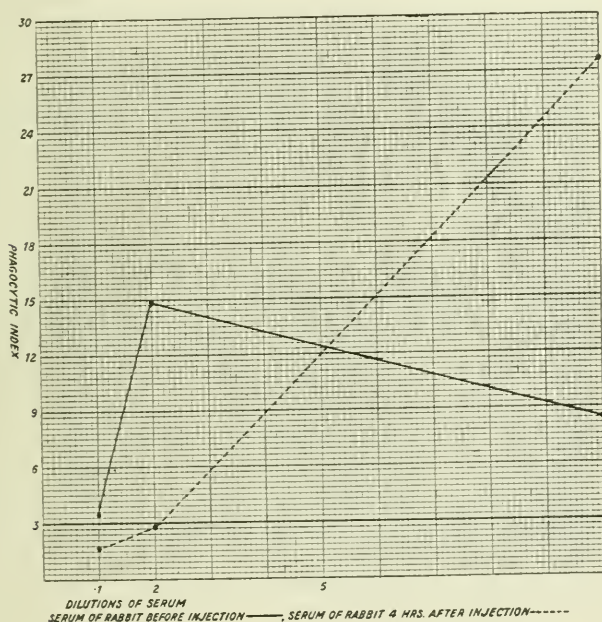


FIG. 5.

usual for the opsonic value of the normal serum to rise to a certain extent, to fall again on further dilution (Dean, G.¹⁵). In the case of the serum of an animal which had received an injection of nuclein the opposite occurred, a very marked fall having taken place even in dilutions of 1 in 5. Now, it is known that in the case of an immune serum, where there is a large increase in the thermostable opsonin, a very extensive dilution indeed is required before the opsonising power

of the serum is destroyed. Seeing that in every experiment recorded here, where an increase in the opsonin has occurred after the injection of nuclein, this increased opsonising power of the serum has been destroyed on diluting the serum with normal saline to 1 in 5 or 1 in 10, it may be concluded that we are not dealing with an increase in the normal thermostable opsonin. In Chart V. it will be seen that in a 1 in 5 dilution the phagocytic index of the serum after injection has fallen below that of the serum before injection, despite the fact that in the undiluted state it is more than three times as great. This question will have to be worked out in greater detail, and the part played by electrolytes in this phenomenon suggests itself for examination.

If, then, there is no alteration in the thermostable opsonin, it appears probable that we may be dealing with an increase in the complement content of the serum, and that this is responsible for the increased opsonic power of the serum. Bulloch (1901¹⁶), thinking it probable that substances which produced a leucocytosis might also increase the complement content of the serum, examined the effect of sodium cinnamate in this respect and claimed to have shown a rise in the quantity of complement. More recently, however, Busse (1908¹⁷) has failed to demonstrate this increase. It is possible, however, that the substance present in these preparations of nuclein and nucleic acid which produces the leucocytosis also gives rise to an increase in the complement, or that these two effects are due to two different substances acting side by side.

Should the injection of nuclein prove to be without effect on the complement content of the serum, we shall be left to conclude that the effect is due merely to some physical-chemical change which is favourable to phagocytosis. Then, again, the question of whether or not this phenomenon takes place *in vitro* must be gone into, and it is interesting to note in this respect that Guerrini (1908¹⁸) claims to have shown that this does occur, but he attributes it to a stimulating action on the part of the nuclein on the leucocyte. In the single experiment performed by Ledingham and Bulloch (1906⁹), nuclein was found to be without effect on phagocytosis *in vitro*. With these points of view in mind it is proposed to continue this study of the effect of these substances on the normal antibody.

CONCLUSIONS.

1. Nuclein alone, out of the three leucocytosis-producing substances examined, brings about a rise in the opsonic index.
2. This rise is first in evidence 20 to 30 minutes after the injection, is still marked $7\frac{1}{2}$ hours after, but has completely disappeared in 24 hours.
3. The increased opsonic activity of the serum is readily destroyed both by heating for 30 minutes at 56° C. and by diluting with normal

saline. It would appear therefore that there is no increase in the thermostable opsonin normally present in the serum.

4. This effect is not produced by all preparations of nuclein or nucleic acid, as, out of the four examined, two were found to be without effect. Seeing that these two latter preparations were the purest of the four, it would appear probable that this effect is not due to the nucleic acid radicle but to the presence of some impurity.

My thanks are due to Dr. Ledingham, at whose instigation this work was commenced and who has directed my work throughout; also to Dr. Hartley for his advice on questions of a chemical nature.

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THE INFLUENCE OF METABOLIC FACTORS
IN BERI-BERI. PART I. THE EFFECT OF
INCREASING THE CARBOHYDRATE RATION
ON THE DEVELOPMENT OF POLYNEURITIS
IN BIRDS FED ON POLISHED RICE.

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(From the Lister Institute.)

It is now taken as definitely settled that the disease beri-beri is essentially privatory in origin, resulting from the deficiency in the food supply of a substance or substances the precise nature of which is at present unknown. If this simple explanation were complete it would follow that the more rapid and thorough the withdrawal of the active substance, the speedier would be the onset of the disease and the more severe its effects.

Maurer (1907) found, however, that when fowls were fed on polished rice those birds receiving the largest quantity of rice were the first to develop symptoms of polyneuritis and that by feeding small amounts of rice the disease could be postponed for a long time.

Chamberlain, Bloombergh, and Kilbourne (1911) on the other hand found that birds consuming large rations of polished rice remained free from polyneuritis for a longer time than birds eating little or no rice at all.

Cooper (1913) however obtained results similar to Maurer, finding that pigeons eating very small amounts of polished rice developed symptoms of polyneuritis in from 20 to 33 days, while those birds receiving daily rations of rice exceeding 1/30th their initial body-weight fell ill in from 9 to 26 days. No constant difference however was noticed in the periods elapsing before the appearance of polyneuritis in the

case of the pigeons receiving daily rations of polished rice varying from 1/11th to 1/30th the initial body-weight.

Caspari and Moszkowski (1913) made observations of a similar nature. They found that pigeons fed on hens' eggs, grape-sugar, and salt remained quite healthy and increased in weight, but pigeons fed on the same rations of egg, sugar, and salt with the addition daily of polished rice developed polyneuritis.

Funk (1914) has still more recently obtained similar results to Maurer (1907) and Cooper (1913), having found that birds fed on large rations of polished rice develop polyneuritis in a shorter time than birds fed on small rations. He also fed series of pigeons on dietaries containing respectively excess of starch, sugar, protein, and fat, and found that the birds receiving the large rations of starch were the first, while those receiving excess of fat were the last, to develop polyneuritis, the birds fed on large rations of sugar and protein falling ill intermediately.

It is thus seen that when the food ration is increased by the addition of material, *e.g.* polished rice, with a very low content of anti-neuritic substance, although the daily allowance of active substance is slightly increased, the development of polyneuritis is actually accelerated.

Similarly birds deprived of everything but water and therefore receiving no anti-neuritic substance at all may live longer than birds fed on a diet, *e.g.* polished rice with a very low content of the active substance, and may die finally without any indication of polyneuritis, although occasional cases of polyneuritis with fatty nervous degeneration were observed by Chamberlain, Bloombergh, and Kilbourne (1911) in fowls. The animal supplied with a little of the substance thus succumbs to the disease, but that receiving none at all may fail to do so. It has been suggested that the starved animal drawing upon its own tissues for a supply of combustible material obtains an adequate amount of anti-neuritic substance in the process of tissue destruction. This is a plausible explanation of the phenomenon, but it does not account for the results of Experiments VI, X and XI (in the present paper) which show first that birds fed on rations equal to 1/20th of their body-weight of washed polished rice lost as much as 40 per cent. in weight and yet developed polyneuritis, and, secondly, that a supply of anti-neuritic substance adequate for the organism provided that the carbohydrate ration was maintained at a certain magnitude was unable to avert polyneuritis when the carbohydrate ration was increased.

Certain facts concerning human beri-beri are parallel to these experimental observations made in the bird. Thus paradoxically, it has been

found by nearly all observers (*Cause and Prevention of Beri-Beri*, Braddon, 1907, p. 256) that in epidemics of beri-beri the well nourished are the first to succumb and are actually more liable to the disease than the underfed. Similarly, under natural conditions men, owing to their larger energy output, partake of polished rice much more freely than women and are more liable to beri-beri. In institutions however where men and women have the same fixed rations the sexes are equally susceptible to the disease. Again, in prisons and asylums in which the dietary has been varied from time to time, every increase in the polished rice component of the diet, the rations of other foodstuffs remaining the same, was attended by increased beri-beri. From these observations Braddon drew the conclusion that in rice-eating communities the extent and severity of beri-beri vary directly with the quantity, absolute or relative, of polished rice consumed.

Admitting, then, that in the production of beri-beri deprivation of a certain substance is an essential factor, it is of obvious importance to ascertain the precise rôle which the active substance plays in metabolism, and to determine, if possible, the quantitative relations which the intake of it must bear to the other components of the dietary, *i.e.* to obtain some information as to the composition of diets best adapted for promoting, first, a good absorption, and, secondly, an economical utilization of the active substance and yet adequate for maintaining the organism in nutritive equilibrium.

With this end in view and also on account of its special importance in the prevention of beri-beri the effect upon the development of polyneuritis in birds of increasing the carbohydrate ration of a dietary containing a certain amount of the anti-neuritic substance has been studied quantitatively and the results obtained are set forth in this communication.

Experiments I to XII were carried out by one of us (W. L. B.) at Seremban, Federated Malay States, during the years 1909-11 and the results are borne out by the remaining experiments which were undertaken by the other of us (E. A. C.) at the Lister Institute during 1913.

I. *The effect of rice-diets upon the health and body-weight of birds.*

Experiment I. Padi as an exclusive food for fowls.

Padi, the whole unhusked rice grain, containing at the best about seven per cent. of protein and one per cent. of fat, is by no means a good food for birds, but it was found that fowls fed daily on 1/20th their body-weight of padi could be maintained in fair health for from 60 to 100 days,

after which they usually died without however any indication of polyn neuritis, although some had lost considerably in weight.

TABLE I.

Bird	Change in weight by 30th day
1	- 20 per cent.
2	+ 18 "
3	- 37 "
4	+ 10 "
5	- 23 "
6	- 23 "
7	- 24 "
8	+ 27 "
9	- 7 "
10	- 7 "

Experiment II. Cured or unpolished rice.

Cured or parboiled unpolished rice contains all the elements of the original padi except the husk and coloured layer of the pericarp, which are rubbed off together after the process of boiling or steaming and subsequent drying. This rice is about 10 per cent. richer in protein and fat than padi.

Four fowls fed on 1/20th their body-weight of this rice remained free from polyn neuritis, but died on the 38th to 78th day of the experiment of intercurrent disorders.

Six birds fed on rations of "cured" rice varying from 1/10th to 1/26th initial body-weight with two exceptions maintained their weight for four weeks as indicated below.

TABLE II.

Bird	Ration	Change in weight by 26th day
85	1/26th body-weight	0
86	1/13th "	- 17 per cent.
87	1/10th "	0
88	1/16th "	0
89	1/15th "	- 17 per cent.
90	1/12th "	0

Experiment III. "Fresh" rice.

Fresh rice, or partly polished hand-pounded rice prepared by the Malay natives for home use, retains portions of the inner pericarpal layer or silver skin, as well as much of the aleurone layer which contains about half the total proteids and fats of the seeds. Hence the rice

has a food value almost as good as that of padi or parboiled rice. Fowls fed naturally on this rice, *i.e.* on rations less than 1/20th body-weight, maintained body-weight and remained healthy for at least one month, as shown in the following table.

TABLE III.

Bird	Change in weight by 30th day
55	0
56	+ 35 per cent.
57	0
58	0
59	- 40 per cent. (Died, but no polyneuritis)
60	0

The rice, such as was used in the above experiments, in which the surface layer of the endosperm is wholly preserved, contains on an average 7 per cent. of protein and 80 per cent. of carbohydrate. Roughly it may be said therefore that physiological balance could be maintained on a diet of which protein composed about 1/300th and carbohydrate 1/25th the body-weight of the birds when sufficient anti-neuritic substance was provided.

The following experiments illustrate what happens, first, when the supply of anti-neuritic substance is reduced, the other food materials remaining constant, and, secondly, when the intake of active substance is kept constant and the carbohydrate ration increased.

Experiment IV. Polished Siam Rice, 1/20th body-weight.

Fowls fed on the above ration developed polyneuritis in from 24 to 69 days, six birds falling ill on the 24th, 25th, 42nd, 43rd, 47th, and 69th days respectively.

It is seen that this rice induced polyneuritis somewhat slowly, but a comparison of the results with those obtained in Experiments I to III indicates that its content of anti-neuritic substance was much smaller than that of padi, cured, or fresh rice.

Experiment V. Washed polished Siam rice, 1/20th body-weight.

In this experiment the rice was first soaked in excess of water for 24 hours. Fowls fed on the above rations developed polyneuritis in from 10 to 35 days, six birds falling ill on the 10th, 13th, 14th, 24th, 32nd, and 35th days respectively. It is seen that this rice induced

polyneuritis more rapidly than the original polished rice, indicating that it had been to a considerable degree deprived of active material by the process of soaking.

Experiment VI. Washed polished Siam rice, 1/20th body-weight.

In this experiment the rice was soaked in excess of water for 48 hours before feeding, and fowls fed on the usual ration developed polyneuritis in 15 to 17 days (15, 15, 15, 15, 16, 17). By the prolonged washing the content of anti-neuritic substance was thus still further diminished if not wholly cut off, and polyneuritis was greatly accelerated. The loss in weight in the case of these birds amounted to as much as 25 to 40 per cent. at the end of the fortnight and in general exceeded anything observed in the other series as shown in the following table. This indicates apparently that the changes culminating in the final break-down (polyneuritis) begin immediately with the deprivation of the protective substance.

TABLE IV.

Bird	Change in weight at end of 14 days			
	Padi 1/20th body- weight ration	Cured rice 1 10th—1/20th	Fresh rice (less 1/20th body-weight) (partly-polished)	Siam pol. rice washed 48 hrs. 1/20th body-weight
1	-30 %	+ 4 %	0	-40 %
2	0	- 4	+20 %	-36
3	-35	- 5	+ 5	-23
4	0	- 6	0	-25
5	-23	-14	-20	-40
6	-30	- 5	+ 3	-35
7	-26			
8	-10			
9	- 7			
10	-10			

The following experiment corroborates these results and also illustrates a point of great practical importance.

Experiment VII.

Ten fowls were fed on 1/20th their body-weight of cured or parboiled rice which had been previously soaked in cold water for 24 hours. Nine of the birds developed polyneuritis in from 13 to 39 days as will be shown in Table V, although birds fed on the same ration of the original parboiled rice had not developed polyneuritis on the 38th to 78th days.

The custom of soaking rice before cooking and then discarding the water has been practised in some institutions and has actually been accountable for severe epidemics of beri-beri amongst the inmates.

In the case of two of the 10 fowls fed on the soaked parboiled rice post-mortem examination revealed general oedema, hydropericardium, and distension of gall bladder, appearances rarely occurring in avian beri-beri. This suggests that further experiments with this rice may lead to the differentiation of the factor which determines the occurrence of oedema in human beri-beri.

The effects of the various rices upon the health of birds are classified in the following table.

TABLE V.

Rations 1/20th body-weight.

No.	Padi	Cured Rice (unpolished rice)	Fresh Rice (partly polished by hand)	Polished Siam rice			Cured rice Soaked for 24 hrs.
				1	2	3	
				Original	Soaked in excess of water 24 hrs. Polyneuritis appeared on	Soaked for 48 hrs.	
1	Died on 67th day. No polyneuritis	Died on 38th day. No polyneuritis	All healthy after 4 weeks	24th day	10th day	15th day	13th day
2	Died on 72nd day. No polyneuritis	Died on 47th day. No polyneuritis	...	25th "	13th "	15th "	15th "
3	Died on 93rd day. No polyneuritis	Died on 54th day. No polyneuritis	...	47th "	14th "	16th "	18th "
4	Died on 96th day. No polyneuritis	Died on 78th day. No polyneuritis	...	42nd "	24th "	15th "	26th "
5	Still healthy on 100th day	...	...	43rd "	32nd "	16th "	32nd "
6	...	...	...	69th "	35th "	17th "	39th "
7	...	...	...	...	...	...	16th "
8	...	...	...	...	...	...	23rd "
9	...	...	...	...	...	...	25th "
10	...	...	...	...	...	...	26th "
11	...	...	...	...	...	...	34th "
12	...	...	...	...	...	...	Died on 63rd day without polyneuritis

Experiments IV to VII prove no more than is well known that the consumption of rice deprived of a certain substance induces beri-beri. The observations however have value according to the light in which they are regarded. In Experiments I to III the birds were fed on a ration containing a definite amount of starch and a definite amount of

anti-neuritic substance, the association of which with the starch prevented polyneuritis. In Experiments IV to VII it is seen that the ration of carbohydrate remaining constant, while the supply of active substance was reduced or withdrawn, beri-beri followed and the effect varied directly with the extent of withdrawal.

The first three experiments furnish a rough index of the normal quantity of anti-neuritic substance necessary daily to maintain physiological balance in a fowl when a normal ration of food is ingested. This quantity is the amount contained in a bulk of padi equal to 1/20th the body-weight of the bird.

Experiments were next made with commercial starch such as is sold for laundry purposes. 10 gms. daily were given alone to fowls of 300 to 500 gms. and the results are tabulated below.

Experiment VIII. Five fowls fed on a ration of 10 gms. of pure starch only, daily.

TABLE VI.

No.	Day, and weight in grammes											Results
	1	4	8	10	12	14	16	18	20	24		
37	525	510	570	600	540	480	433	450	360	375	345	Became weak on 16th day and remained so till 20th but no signs of beri-beri. Food changed to padi but died 24th day.
49	375	375	405	360	240	—	—	—	—	—	—	Sickened but no signs of paralysis, died 10th day.
50	300	270	365	300	300	240	225	173	—	—	—	Gradually wasted, on 14th day very weak but no paralysis. Died 16th day.
51	480	435	465	450	390	390	463	345	—	—	—	Wasted and weak but able to run, transferred on 16th day, recovered.
52	465	450	465	413	435	390	360	360	345	300	—	On 20th day very thin and weak but no paralysis; somewhat somnolent. Food changed. Recovered.

Although considerable loss of weight took place in each of these birds, none of the ordinary symptoms of avian beri-beri were noted up to the time when they became critically ill.

The condition seemed to be (after the first few days) one of simple starvation. It appears probable that, although at first some of the starch given was digested, as the increases of weight in some of the birds indicates, later on absorption ceased, possibly for mechanical reasons—the starch being apt to make a solid mass in the crop and duodenum. As will be seen later exclusive diets of glucose induced polyneuritis in birds and the addition of sago to a diet consisting of polished rice and yeast accelerated the development of the disease, Expt. XV (p. 345).

Experiment IX. Six fowls were fed on a daily ration of 30 gms. starch plus 1 gm. of egg-yolk.

TABLE VII.

No.	Day, and weight in grammes							Remarks
	1	9	11	13	15	17	19	
63	570	480	540	480	420	150	330	Died on 19th day. No paralysis.
65	570	540	540	480	420	—	—	Died on 15th day. No paralysis.
100	660	660	630	540	540	—	—	Died on 14th day. No paralysis.
94	1200	1050	1050	1020	1020	1020	900	Sick on 19th day, legs paralysed.
1	1370	1320	1290	1260	1200	1040	990	Died on 19th day. No paralysis.
10	1500	1340	1340	1380	1350	1350	1260	Quite well and strong on 19th day.

The egg-yolk in this experiment was mixed thoroughly into a paste with the starch and a little water, the mixture being given in pills. Although there were four deaths, in none of the four were characteristic signs of beri-beri observed previously.

One bird, however, developed beri-beri in the subacute form and one remained except for loss of weight quite well at the end of the experiment.

Cooper and Funk (1911) obtained somewhat similar results, pigeons fed on 1/10th to 1/40th daily rations of Coleman's starch, potato-starch, inulin, dextrin, and cane sugar in some cases developed typical symptoms of polyneuritis with considerable fatty degeneration in sciatic nerves and spinal cords. Many of the birds however only exhibited signs of weakness and then died, but even in these there was evidence of fatty degeneration. In all cases the deficiency in the diet of the anti-neuritic substance had evidently made itself felt, but in some cases at the time of death the depletion of the body store of active substance had only proceeded far enough to cause some degree of fatty degeneration, and was not sufficient to induce the development of the typical neuritic symptoms. The proportion of undoubted cases of polyneuritis is given below.

TABLE VIII.

Diet	No. of cases	Distinct cases of polyneuritis	Cases of weakness only
Coleman's starch	3	1	2
Potato starch (pure)	6	1	5
Inulin	3	2	1
Cane sugar	7	1	6
Dextrin	8	5	3

The frequent failure of such carbohydrate diets to induce typical polyneuritis is surprising in view of the facts that the symptoms regularly develop in birds fed liberally on polished rice and even when polished rice or sago is added to a basal ration containing a minimum of the anti-neuritic substance (Experiments X to XV). In the case of starch, as already pointed out, the condition would appear to be one of simple starvation. The case of cane-sugar however offers more difficulty, as it would seem unlikely that saccharose escaped inversion in the alimentary canal. The excreta were however not examined for the presence of saccharose.

The results with dextrin show that exclusive diets of this carbohydrate frequently induced distinct symptoms of polyneuritis. This may be due to the fact that dextrin dissolves in water at ordinary temperatures and would be readily hydrolysed to glucose in the alimentary canal, so that the birds were thus called upon to metabolize large amounts of carbohydrate and depletion of their store of anti-neuritic substance rapidly took place.

Experiments with glucose have since been carried out, and it has been found that of four birds fed exclusively on aqueous solutions of this carbohydrate three developed typical symptoms of polyneuritis, while the fourth only exhibited symptoms of weakness with however considerable fatty degeneration in the sciatic nerves.

II. *The effect of increasing the carbohydrate ration upon the time elapsing before onset of polyneuritis in birds.*

The succeeding experiments show what happens when the intake of anti-neuritic substance is maintained constant, but the proportion of food-material is increased.

Experiment X.

(a) Six fowls weighing about 600 gms. each were fed on a ration of 30 gms. of padi daily (1/20th body-weight) for 45 days.

(b) Another six fowls were fed on the same ration of padi as the above plus 90 gms. of water-extracted unpolished rice (1/6th to 1/7th body-weight).

The birds of series (a) maintained their body-weight and were quite healthy at the end of 45 days, but those included in series (b) all developed polyneuritis in from 10 to 16 days.

Experiment XI.

(a) Ten fowls weighing about 600 gms. each were fed on $22\frac{1}{2}$ gms. padi daily (about 1/25th body-weight).

(b) Ten other fowls received the same ration of padi and in addition 54 gms. of polished Siam rice daily (1/10th body-weight approximately).

Nine of the birds fed on padi only were strong and healthy at the end of 30 days, three of them gaining in weight and six losing in weight. The 10th bird escaped early in the experiment.

TABLE IX.

(a) Padi only, $22\frac{1}{2}$ gms. daily.

No.	Day, and weight in grammes							Effect of diet and % change by 30th day
	1	5	8	10	21	27	30	
1	600	510	472½	450	372½	—	480	- 20 %
2	600	630	570	510	630	—	705	+ 18 %
3	600	480	450	360	420	—	375	- 37 %
4	420	500	465	375	450	—	465	+ 10 %
5	570	600	540	420	450	—	442½	- 23 %
6	570	480	472½	390	420	—	442½	- 23 %
7	540	450	420	360	435	—	412½	- 24 %
8	495	600	540	537½	360	—	630	+ 27 %
9	420	540	510	390	—	—	—	Escaped
10	540	540	480	420	540	—	502½	- 7 %

All healthy on
30th day.

TABLE X.

Padi $22\frac{1}{2}$ gms. + Polished rice 54 gms. daily.

No.								Effect of diet and % change in weight by end of exp.	
	1	5	8	10	21	27	30		
11	660	690	600	592½	600	—	615	Beri-beri 28th day. Died 29th day	- 7 %
12	540	570	510	510	540	480	—	Beri-beri 19th day. Died 29th day	- 11 %
13	390	480	420	390	375	—	—	Died suddenly at night 20-21st day	0
14	510	540	510	510	570	—	615	Beri-beri 28th day. Died 29th day	+ 20 %
15	570	570	510	495	555	—	555	Died acute beri-beri 30th day	0
16	630	480	480	435	472½	480	—	Beri-beri 25th day. Died 29th day	- 24 %
17	480	510	450	447½	420	—	420	Beri-beri 26th day. Died 29th day	- 12 %
18	600	570	540	525	600	—	735	Died acute beri-beri 30th day	+ 22 %
19	600	630	600	600	600	—	—	Died suddenly 20th day	0
20	570	540	480	450	480	—	—	Died acute beri-beri 30th day	- 16 %

The birds receiving polished rice as well all developed polyneuritis in from 20 to 30 days, five losing in weight, three remaining constant, and two gaining in weight. The results are set forth in detail in Tables IX and X.

[All these birds showed signs of early beri-beri—dullness, tremors, somnolence, and weakness on 18th to 19th day.]

The amount of anti-neuritic substance contained in 1/20th to 1/25th ration of padi was sufficient to prevent polyneuritis in fowls for at least 45 days, when fed exclusively on the diet (Experiment X), yet, although by the addition of washed polished rice to the diet the amount of active substance was actually slightly increased, the effect of the addition was to produce polyneuritis in less than 30 days. The conclusion clearly emerges that the amount of active substance required by the organism stands in some quantitative relation to the carbohydrate ration which has to be metabolized.

Experiment XII.

In this experiment birds were fed on small and large rations of water-extracted polished rice.

TABLE XI.

Series (a). 1/50th body-weight ration of rice for 1st 5 days, subsequently 1/12th body-weight.

Effect of diet		% change in wt. by end of exp.
1.	Polynuritis on 15th day	- 18 %
2.	 15th "	- 20 %
3.	 14th "	- 10 %
4.	 20th "	- 40 %
5.	 15th "	- 13 %
6.	Healthy for 24 days	0

Series (b). 1/5th body-weight ration of rice.

1.	Polynuritis on 11th day	- 20 %
2.	 16th "	- 16 %
3.	 15th "	- 27 %
4.	 14th "	- 7 %
5.	 11th "	- 9 %
6.	 15th "	- 3 %

The results show that, unlike what happened in Experiments X and XI, in which the anti-neuritic substance was provided in the diet, there was practically no difference in these two series as to time of onset of polyneuritis. It may be inferred that the lower scale of ration was already sufficient to produce the maximum effect in depletion of

TABLE XII. EXP. XIII.

Series I. Daily rations. Polished rice 1/25th initial body-weight. Yeast 1/2500th.

[illegible]

Polished rice 1/10th initial body-weight. Yeast same as above.

[illegible]

the body store of active substance. Cooper (1913) obtained similar results. It was found that rations of polished rice varying from 1/30th to 1/10th of the body-weight induced polyneuritis in pigeons in about the same time (9 to 26 days), but when the rations fed were below 1/30th polyneuritis was delayed and did not develop until 21 to 33 days had elapsed. The maximum effect is evidently induced by a ration of carbohydrate of about 1/30th the body-weight of the bird. In quantitative investigations of the effect of various conditions upon the rate of onset of polyneuritis it is thus of importance to supply the organism with the minimum amount of vitamine and not to rely merely on the traces of active substance stored in the tissues. This point has frequently been overlooked in the past.

Experiments XIII to XV.

This series of experiments differs from those previously detailed in that all the birds were fed upon polished rice or sago with the addition of a constant amount of dried brewer's yeast sufficient to prevent polyneuritis for from 30 to 50 days when normal rations of carbohydrate were fed. By varying the carbohydrate ration and maintaining the supply of yeast constant, it was thus possible to study quantitatively the effect of increasing the carbohydrate component of the dietary upon the time elapsing before the onset of polyneuritis.

Experiments with pigeons.

The pigeons were first of all fed for three weeks on constant daily rations of whole barley equal to 1/20th their initial body-weight.

Experiment XIII.

Six pigeons were then fed daily on amounts of polished rice and yeast equal respectively to 1/25th and 1/2500th their initial body-weight. For birds weighing 350 gms. the daily quantities of rice and yeast were therefore 14 gms. and 0.14 gm. A second series of pigeons (eight) received daily the same proportion of yeast but two and a half times as much polished rice as the above birds, the rations of rice being thus 1/10th the initial body-weights.

Artificial feeding was employed in all the experiments.

Experiment XIV.

In this experiment the daily rations were as follows :

Series I. (Three birds.) Polished rice = 1/20th initial body-weight.

Yeast .. = 1/3500th ,, ,,

Series II. (Three birds.) Polished rice = 1/10th ,, ,,

Yeast .. = 1/3500th ,, ,,

For pigeons weighing 350 gms. the daily rations were therefore

Polished rice, $17\frac{1}{2}$ and 35 gms. respectively.

Yeast .. 0.1 gm.

TABLE XIII. EXP. XIV.

Series I. Daily ration 1/20th initial body-weight polished rice and 1/3500th body-weight yeast.

No. of pigeon	Effect of diet upon health of birds	Body-weights							% change in wt. by end of exp.	% change in wt. by 18th day, the average time taken by the birds on large ration to develop polyneuritis
		Initial	Day 5	16	26	37	41	53		

Series I.

103	Polyneuritis on 37th day	390	390	380	350	300	—	—	-23 %	- 2 %
104	Weakness in limbs on 44th day. Polyneuritis on 53rd day	340	320	330	290	250	250	230	-32 %	- 3 %
			Day 7	14	21	28	37			
141	Polyneuritis on 32nd day	340	370	330	320	280	290	...	-15 %	- 3 %

Series II.

145	Polyneuritis on 15th day	310	—	370	—	—	—	...	+19 %	+19 %
146	Polyneuritis on 16th day	300	370	350	—	—	—	...	+16 %	+16 %
148	Weakness in limbs on 22nd day. Polyneuritis on 26th day	320	400	350	320	—	—	...	0	+ 4 %

Experiment XV (with chickens).

The birds were first of all fed for three weeks on whole barley, the daily rations being equal to 1/20th their initial body-weight.

Five chickens were then fed on 1/45th their initial body-weight of polished rice and 1/2500th of dried pressed yeast, a chicken of 1450 gms. weight thus receiving daily 32 gms. of polished rice and 0.6 gm. of yeast.

Five other chickens received daily the same rations of rice and yeast as the above, but in addition 1/45th their initial body-weight of sago. Sago was used in this experiment instead of polished rice to facilitate

artificial feeding, as sago passes the crops of birds much more readily than rice¹.

The results of Experiment XIII show that of six pigeons receiving daily $1/25$ th their initial body-weight of polished rice with $1/2500$ th of dried pressed yeast, three remained quite healthy for 95 days and even at the end of that time showed no signs of polyneuritis, one was healthy at the end of 47 days, and two developed polyneuritis in 39 and 52 days. The eight pigeons fed daily on $1/10$ th their body-weight of polished rice with the same amount of yeast as the above birds, however, all developed polyneuritis in from 22 to 46 days.

Experiment XIV shows that pigeons fed on daily rations of polished rice equal to $1/20$ th their initial body-weight with $1/3500$ th of yeast developed polyneuritis in from 32 to 44 days, but those fed on $1/10$ th their body-weight of rice with the same amount of yeast became ill in from 15 to 22 days.

Pigeons fed exclusively on the same polished rice in amounts equal to $1/10$ th to $1/30$ th their initial body-weight daily developed polyneuritis in from 9 to 26 days (Cooper, 1913). It is thus seen that, while the addition of a small amount of yeast to the polished rice diet could prevent polyneuritis in pigeons for a considerable time when the daily ration of rice did not exceed $1/20$ th the initial body-weight, when the ration of rice was increased to $1/10$ th, the yeast had little protective effect and the period elapsing before the onset of polyneuritis did not greatly exceed that elapsing when the diet was exclusively polished rice.

Experiment XV (with chickens) shows that of the five birds fed daily on $1/45$ th their initial body-weight of polished rice with 0.5 to 0.7 gm. of yeast one was still healthy on the 100th day of the experiment, three developed polyneuritis on the 94th to 97th days, and one on the 62nd day. The other five birds receiving in addition $1/45$ th their initial body-weight of sago daily fell ill with polyneuritis in from 13 to 31 days. As in the case of the previous experiments with pigeons, doubling the daily ration of carbohydrate thus greatly accelerated the development of polyneuritis.

The eight pigeons (Experiments XIII and XIV) fed on the small rations all lost in weight, while of the 11 pigeons fed on large rations

¹ It should be mentioned that during the first week of the experiment the rations of carbohydrate employed were somewhat larger, being $1/30$ th and $1/15$ th the initial body-weight of the birds. As some difficulty was met with in feeding these large amounts, the rations finally employed were as above.

(1/10th their body-weight) four gained in weight, three remained constant in weight, and the remaining four lost to as great an extent as some of the birds fed on the small rations and yet developed polyneuritis earlier. These results are of interest as they indicate that a dietary deficient in the anti-neuritic substance can sometimes maintain body-weight and even lead to increase in weight. Similarly, in Experiment XV of the five chickens fed on the large ration, three maintained their body-weight, but two lost in weight to as great an extent as the birds fed on the small ration and again developed polyneuritis considerably earlier.

The results thus confirm the earlier observations that the amount of anti-neuritic material required by the organism is not constant and independent of the quantity of food provided, but as the ration of carbohydrate is increased the demand of the organism for the active substance is also increased and if this be not adequately met, polyneuritis follows. When the carbohydrate ration was doubled (as in Experiment XV) by the addition of sago to a diet consisting of polished rice and yeast, the development of polyneuritis was accelerated considerably more than two-fold. Thus, when daily rations of polished rice and yeast equal respectively to 1/45th and 1/2500th the initial body-weight of the birds were fed, polyneuritis did not occur for nearly 100 days, but when the diet was supplemented by an equal quantity of sago, symptoms of polyneuritis appeared in from 13 to 31 days.

In this experiment the excreta of the fowls were collected daily, dried at ordinary temperatures by means of an electric fan, and the amount of undigested starch estimated by hydrolysis with acid and determination of the quantity of sugar produced by reduction (Bertrand) and polarimetric methods. The results, given in Table XV, were obtained.

The results show that in the case of the birds fed on the small rations the percentage of starch digested varied from 95 to 100 per cent., and in the case of the birds fed on the large rations from 90 to 100 per cent., the greater part of the starch fed thus being hydrolysed in the alimentary canal. Only small amounts of sugar could be detected in the excreta, and no dextrin was found at all.

Cooper (1914) found that alcoholic extracts of the excreta of a chicken fed on grain and the faeces of a rabbit fed on cabbage cured polyneuritis in pigeons, so that a possible explanation of the effect of large rations of starch in accelerating polyneuritis was that a portion of the starch was not digested, and interfered with the absorption of the

TABLE XV.

	Bird	Period during exp.	Starch fed	Starch excreted	Period during exp.	Starch fed	Starch excreted
Small rations	A	5th-11th day	204	5.0	12th-18th day	156	2.5
					19th-25th „	156	0
	B	„	228	2.5	—	—	—
	C	„	228	7.0	12th-18th „	162	9.0
					19th-29th „	250	6.0
Large rations	D	„	228	1.0	12th-25th „	325	4.0
	E	„	207	2.0	12th-33rd „	492	0
	F	„	346	4.5	—	—	—
	G	„	413	7.0	12th-33rd „	965	5.0
	H	„	476	8.0	12th-25th „	674	0
	I	„	325	3.5	12th-18th „	351	34.0
					19th-25th „	351	16.0
	K	„	636	3.1	12th-18th „	468	36.0
					19th-29th „	720	8.0

anti-neuritic substance supplied in the diet. As however the amount of starch excreted by the fowls in Experiment XV was so small, it seemed improbable that the above explanation could hold at any rate for the particular rations employed in that experiment. Nevertheless it was decided to attempt to ascertain whether the anti-neuritic substance could be detected in the excreta. Pigeons were fed daily on 1/25th their body-weight of polished rice to which was added varying amounts of the pulverised dry excreta of chickens A, E (small rations of rice) and G, I (large rations).

Assuming only half the amount of active substance fed to the chickens was absorbed, the amount contained in the excreta administered should have prevented polyneuritis in the pigeons for 39 to 95 days (see Experiment XIII).

Experiment XVI.

TABLE XVI.

Pigeon	Amount of dry excreta fed daily	Effect on pigeon
1	3.5 g. of chicken A (small ration)	Weakness in wings and limbs 25th day. Polyneuritis acute 32nd day.
2	1.5 g. of chicken E (small ration)	Polyneuritis on 22nd day.
3	3.5 g. of chicken G (large ration)	Weakness in wings and limbs on 27th day.
4	4.5 g. of chicken I (large ration)	Weakness in wings and limbs 25th day. Acute polyneuritis 32nd day.
Pigeons fed on 1/25th body-weight of polished rice only		Polyneuritis in 20-28 days.

The excreta were evidently free from the anti-neuritic substance, and it thus seems that in Experiment XV the rapid onset of polyneuritis in the case of the birds receiving the large ration of carbohydrate was not due to imperfect absorption of the active material.

Experiments were next carried out with the object of ascertaining (1) whether the active substance is adsorbed by starch and (2) whether chickens digest the starch as readily when the ration is raised to 1/10th their body-weight (the maximum ration employed in the above digestion experiment being 1/20th the body-weight).

To 50 c.c. of a curative yeast extract 20 gms. of potato starch were added, and the mixture was allowed to stand at room temperature with frequent shaking for several days. The minimum curative dose of the solution for neuritic pigeons of about 300 gms. weight was determined before and after the addition of the starch, 10 animals being employed altogether. It was found that 2-3 c.c. of the solution before and after treatment with starch was just sufficient to bring about complete recovery, smaller doses having only a slight ameliorative effect. There was thus no evidence that starch adsorbed the active substance to any appreciable degree.

It seemed still possible, however, that with a much larger ration of starch the absorption of the active substances from the gut might be interfered with.

Four chickens were therefore fed on very large rations of polished rice (1/10th their body-weight) with the addition daily of 4, 6, 2½, and 1½ gms. of dried pressed brewer's yeast respectively. The excreta were collected daily, dried at 20° C. by means of an electric fan, and ground to a fine powder. The birds developed polyneuritis in the remarkably short period of 10 days. The amount of starch in the excreta was estimated and we were surprised to find that of this enormous ration the birds had digested 93 to 98 per cent., the amount of starch excreted per diem varying from 2 to 8 gms. Four pigeons were fed on rations of polished rice equal to 1/25th the body-weight and fixed quantities of the excreta. The amounts given daily were such that assuming provisionally that one half the yeast fed to the original chicken was excreted, they should have contained sufficient yeast (0.15 gm.) to prevent polyneuritis in the pigeons for from 39 to 95 days. (See Experiment XIII.) The pigeons however all developed polyneuritis in from 21 to 30 days, while control birds fed on the same ration of polished rice without the addition of excreta fell ill in from 20 to 28 days.

The addition of the excreta to the rice diet thus neither delayed nor

prevented polyneuritis in the pigeons, and this shows that the rapid onset of polyneuritis which results from the consumption of diets containing large rations of polished rice is not due to interference with the absorption of the anti-neuritic substance.

The main conclusion to be drawn from these experiments is therefore that the amount of anti-neuritic substance required by the organism increases with the quantity of carbohydrate *metabolized*. For the maintenance of health the intake of active substance must therefore be adjusted, as so to stand in some quantitative relation to the amount of carbohydrate included in the diet.

This fact is of practical importance in the prevention of beri-beri, as attention must evidently be paid not only to the absolute amount of anti-neuritic foodstuff incorporated into the dietary, but also to the proportion which this bears to the total carbohydrate ration. The exact ratio which must be maintained has not yet been determined, but the results so far obtained show that when the carbohydrate ration was doubled polyneuritis was induced more than twice as rapidly. Evidently then for the prevention of beri-beri the proportion of the anti-neuritic foodstuff in the diet must be maintained as high as possible, and large rations of foodstuffs deficient in the active substance carefully avoided.

Some minor points arising out of the results deserve mention. It was suggested previously (Cooper, 1914) that the curative action of extracts of excreta was due in some degree to the capacity of bacteria growing in the intestine to synthesize the anti-neuritic substance. Extracts of 2 gms. of *B. coli* (dry) however had no effect upon neuritic pigeons, but this could not be regarded as conclusive evidence, as so small a weight of bacilli was used. As, however, excreta are very rich in bacteria, the failure of as much as 1.0 to 5.0 gms. of excreta daily to prevent or even delay polyneuritis in pigeons fed on polished rice suggests that bacteria compared with yeast (0.15 gm. daily of which prevents polyneuritis for 39 to 95 days) contain an insignificant amount of anti-neuritic substance.

The results also show that birds can digest enormous rations of starch, amounting daily to 1/10th their body-weight.

SUMMARY.

(1) Chickens fed on 1/20th their body-weight daily of padi, cured or parboiled unpolished rice, or fresh (partly polished) rice, remain free from polyneuritis for at least 28 to 100 days.

(2) Chickens fed on the same ration of polished Siam rice develop symptoms of polyneuritis in from 20 to 70 days.

(3) When the polished rice is soaked in excess of water for 24 hours the birds succumb to polyneuritis in from 10 to 35 days; when the rice is soaked for 48 hours the disease appears still more readily, viz. in 15 days.

(4) Chickens fed on 1/20th their body-weight of parboiled rice which had been soaked for 24 hours also develop polyneuritis in from 13 to 39 days. This fact is of practical importance, as epidemics of beri-beri have been traced to the practice of soaking unpolished rice prior to cooking and discarding the water.

(5) Although birds fed on diets of polished rice, sago, or glucose develop polyneuritis, when fed on commercial starch they often fail to do so and merely lose considerably in weight. An explanation is offered.

(6) While fowls fed on 1/20th their body-weight of padi remain free from polyneuritis for at least 60 to 100 days, when this ration is supplemented by 1/6th the body-weight of washed unpolished rice or 1/10th of polished rice polyneuritis appears in from 20 to 30 days.

(7) On the other hand, chickens fed on rations varying from 1/5th to 1/30th of their body-weight of washed unpolished rice develop polyneuritis in about the same time, 10 to 20 days.

(8) On dietaries composed of rations of polished rice varying from 1/20th to 1/40th the body-weight and of yeast varying from 1/2500th to 1/3500th the body-weight pigeons and chickens do not develop polyneuritis until at least 32 to 100 days have elapsed, but when the carbohydrate ration is doubled by the addition of polished rice or sago the birds fall ill in from 13 to 46 days.

(9) Even when daily rations of polished rice as large as 1/10th the body-weight are fed to chickens together with varying amounts of yeast, 93 to 98 per cent. of the carbohydrate is digested and absorbed and the excreta contain no anti-neuritic substance. This shows that the rapid development of polyneuritis induced by feeding large rations of starch is not due to interference of undigested carbohydrate with the absorption of the active material.

(10) Intestinal bacteria, unlike yeast, contain little anti-neuritic substance.

Conclusion and practical application of the results.

The amount of anti-neuritic substance required by the organism increases with the quantity of carbohydrate metabolized. For the maintenance of health the intake of active substance must therefore

be adjusted, so as to stand in some quantitative relation to the ration of carbohydrate ingested, and it is when this necessary balance is not maintained in the dietary that beri-beri results. Although as ordinarily induced beri-beri can be described not inaccurately as a "deficiency disease," it may thus actually develop when a dietary containing an adequate amount of the anti-neuritic substance is regularly supplemented by a ration of a carbohydrate foodstuff deficient in this essential substance.

This is obviously of great practical importance in the prevention of beri-beri. Attention must be paid not only to the absolute amount of anti-neuritic foodstuff incorporated in the dietary, but to the proportion which this bears to the total carbohydrate ration. The precise relation which must subsist between the supply of active material and the amount of carbohydrate metabolized has not yet been ascertained, nor has it been determined for the other normal components of a dietary, *e.g.* protein and fat. It is therefore advisable to maintain the proportion of anti-neuritic foodstuff in the diet as high as possible, and large rations of foodstuffs deficient in the essential substance should be carefully avoided.

In the preparation of a dietary to obviate beri-beri, it thus becomes necessary to consider not merely its absolute content of anti-neuritic material, but also its total calorific value.

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OBLIGATE ANAEROBES IN THE ALIMENTARY TRACT OF INFANTS.¹

(FIRST COMMUNICATION.)

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(PLATES XIX.—XX.)

THE observations here recorded constitute a preliminary note on the anaerobic flora of the intestinal tract in early life. The technical difficulties in work on obligate intestinal anaerobes are considerable, and, in the present state of our knowledge, results are small in proportion to the amount of laboratory work involved. The present paper therefore will deal chiefly with the methods and technique which have given the best results in obtaining pure cultures of these organisms.

The importance of investigation of bacteria of this class is associated with recognition of their capacity to produce putrefactive decomposition of native protein. It is particularly necessary to determine any possible connection between the action of obligate anaerobes in intestinal putrefaction generally, and in the gastro-intestinal disorders in infancy when the anaerobic flora is most evident in the intestinal tract.

Researches on intestinal bacteriology have not dealt to any marked degree with the group of the obligate anaerobes, and our limited knowledge is confused by diversity of nomenclature and by want of uniformity in the culture and chemical methods used for distinguishing individual types. A notable characteristic of such organisms is their extraordinary pleomorphism, and the striking differences in morphology which are associated with variations in culture media render it easy to confuse microscopical appearances. It is quite possible that this property has caused the same organism to be described as different varieties and so complicated the nomenclature.

¹ Received May 8, 1914.

In the absence of micro-photographs it is not possible to compare the organisms described by different observers or to correlate their results.

Our present knowledge has been supplied chiefly by Continental workers. Tissier (1900⁶) was the first to describe, as the dominant organism in fæces of healthy breast-fed infants, a strictly anaerobic bacillus, retaining Gram's stain, and frequently showing a bifurcated extremity. He gave the name *B. bifidus communis* to this organism, and his description has been accepted as a standard one. His observations were made on infants of all ages from a few hours old up to nine months.

Rodella (1902⁵) described three varieties of anaerobic intestinal bacilli isolated from nine cases aged from six days to twenty-one days. These were all very similar morphologically, and all possessed a terminal knob or spore, which identifies them with the "Köpfchen bacillus" of Escherich. No bifid ends are described. Rodella distinguished them as *Bacillus* I., *B. II.*, and *B. III.*, and based his classification on cultural differences and variation in toxicity and not on morphology. The evidence in favour of the existence of three distinct varieties in so small a series of cases is not very conclusive.

Tissier later (1905⁷) admits the existence of *B. III.* of Rodella as a distinct type, but claims that it disappears after the third or fourth day of life. This statement is open to the criticism that Rodella first isolated the organism from infants nine days old.

Herter (1907²) discusses the morphology of *B. bifidus* in some detail. He considers that it occurs in three forms—(a) A plain bacillary form, which is the most frequent type; (b) a bifid form; (c) a headlet or knobbed form. He has cultivated the bifid and knobbed forms from pure cultures of the simple bacillary organisms, and believes that the headlet or knobbed forms are resistant or spore-forming varieties of *B. bifidus*. The ages of the patients examined are not given, and photo-micrographs are not available. The indication, however, is that the bacillus described by Herter is a special form of that classified by Tissier as *B. bifidus*.

It will be necessary to refer again to the views of these three observers after statement of the details of the present investigation.

For this investigation, examinations have been made of evacuations from the alimentary tract of healthy breast-fed infants in the first ten days of life. Eighty specimens have been collected, usually from twenty hours to four days after birth; and the limits of age were eight hours and ten days respectively.

COLLECTION OF MATERIAL.

Fæcal material was removed from the rectum in a sterilised piece of glass tubing, after washing the anus. It was usually possible to collect meconium during evacuation. Inoculations into the special media used were made immediately, and a portion of the specimen was reserved for making smear preparations.

MEDIA EMPLOYED FOR CULTURE.

A constant difficulty in bacteriological examination of fæces is the selective action of artificial media, which allows certain organisms to out-

grow the others. For example, if a portion of faecal material be inoculated (a) into ordinary nutrient bouillon of alkaline reaction, (b) into such bouillon containing a large amount of bile, and (c) into nutrient bouillon acidified with 1 per cent. acetic acid, and if the sediments of the three tubes be examined, each will show a different microscopical picture, and none will conform to that of a smear made directly from the faeces. This difficulty is increased when obligate anaerobes are sought for in ordinary media, as facultative anaerobes of the coliform type obscure the obligate varieties in every case.

For investigating meconium, with its high content of biliary material, the addition of bile to the media seemed a reasonable provision, and fluid ox-bile, in proportion of 25 per cent., was added to ordinary nutrient bouillon as an experiment. A loopful of meconium was inoculated into the tube, and after twenty-four hours' incubation under anaerobic conditions the sediment was examined microscopically and compared with the sediment in a plain broth tube similarly inoculated and incubated. It was evident that the bile-broth mixture favoured the multiplication of Gram-positive bacilli and inhibited the coliform organisms which were abundant in the plain broth tubes. The results were more marked when 50 per cent. bile was added to nutrient broth, and this proportion was subsequently used.

Tissier mentions the addition of 5 per cent. bile to culture media employed for *B. bifidus*, without marked success. The question of inhibition of coliform organisms by large quantities of bile has been discussed recently by Jordan (1913³).

The media most frequently used had the following composition:—

(a) BILE BROTH, consisting of equal parts of filtered green ox-bile and ordinary nutrient peptone bouillon, to which a small portion of sterilised white of egg was added in cubes.

(b) BILE-ALBUMIN PLATES.—White of egg was beaten up with 25 per cent. green ox-bile, and 10 per cent. water, poured in Petri plates and autoclaved. Twelve plates were made at once, and could be kept sterile for days in a metal cylinder.

(c) BILE-SERUM PLATES, made from horse serum, with 25 per cent. green ox-bile added. These were sterilised and kept in the same way as (b).

(d) BILE-ASCITIC-AGAR SLOPES, made of 2 per cent. agar in a mixture of 25 per cent. bile, 25 per cent. ascitic fluid, and 50 per cent. water.

(e) ORDINARY OX-BLOOD AGAR IN SLOPES.—In all these media the reaction was made faintly alkaline to litmus, but no quantitative estimation of alkalinity was made. The omission is probably an important one, as it seems likely that an optimum degree of alkalinity or acidity can be determined.

Immediately after removing the faecal material two loopfuls were inoculated into 5 c.c. bile broth. These tubes were incubated anaerobically for twenty-four hours in a Bulloch's apparatus (vacuum-pyrogallie-soda). After twenty-four hours, the tubes were removed, films were made from the sediment they contained, and a loopful of the

emulsion was smeared over a bile-albumin plate. The plates were then incubated anaerobically for thirty-six to forty-eight hours. Bulloch's apparatus was used throughout with satisfactory results. No nitrogen or hydrogen gas was introduced, and the degree of anaerobiosis was judged by the tinting of the pyrogallie-soda solution, which was generally very lightly coloured.

After thirty-six hours, the surfaces of the plates were examined for distinctive growths, and twelve to fifteen colonies were examined microscopically. Growth was generally not very profuse, and colonies were well separated. Single colonies, when examined, often showed a mixture of cocci and bacilli and had to be re-plated. Growth is less abundant on bile-serum than on bile-albumin. The former plates were therefore more useful if the original emulsion was dense.

Ready recognition of the anaerobic colonies only developed after considerable practice, and it cannot be said that their appearances in surface growth were very distinctive. Experience showed that two kinds of obligate anaerobes could be distinguished on the surface.

1. *B. aerogenes capsulatus*, which appears in large rounded and heaped-up colonies, with edges slightly ragged, and which often sinks slightly into the medium.

2. An anaerobe to which no name need be attached at present, which grows in flat, dull colonies somewhat sunken in the medium, with well-marked radiations at the edges. The dull appearance is very characteristic in uncontaminated colonies. The two anaerobes were frequently associated in a single colony.

TRANSPLANTATION FROM PLATES TO SLOPED MEDIA.

This has proved the most disappointing stage of cultivation. Slopes of egg-media, glucose agar, and meconium agar were tried, without any success. When bile-ascitic agar and ox-blood agar were used, the results were better, but growth failed in about 75 per cent. of the tubes.

It was found more satisfactory to transfer colonies from the plate to deep glucose agar tubes, by stab inoculation, and obtain slope cultures from these when successful. *B. aerogenes capsulatus* grew readily in glucose agar, usually with abundant gas production and splitting of the medium.

The other anaerobe frequently failed to develop in deep glucose agar stabs; but it was found that tubes in which no growth had occurred often showed anaerobic colonies at the site of the first inoculation, *after a second stab inoculation had been made elsewhere in the medium with some other organism*. This apparently symbiotic mode of growth proved the most satisfactory means of obtaining the unnamed anaerobe in pure culture. For the second stab the most useful organisms were *B. aerogenes capsulatus* and *B. coli communis*. The anaerobe whose

growth had been thus encouraged in stab culture could be subcultured readily from deep glucose agar on to blood-agar slopes.

The principal fluid medium used for subculture was *bile broth*, in which both varieties of anaerobic organisms grew readily in pure culture. In *ordinary glucose broth*, *B. aerogenes capsulatus* grew well; the other anaerobe failed to grow in pure culture, but in this medium grew vigorously in symbiosis with *B. lactis aerogenes*.

RESULTS.

I. Meconium Period.

One specimen examined eight hours after birth proved sterile on culture. In all other cases infection had been established when the specimen was collected. Direct smears made from meconium and stained by Gram's method showed fairly uniform appearances. The bacteria are scanty and are extra-cellular. The forms constantly observed were:—

(a) A large thick deep-staining bacillus with square ends, Gram-positive when stained and frequently exhibiting a clear refractile spore close to one end, but never terminal. This organism could always be demonstrated in smears, and multiplied rapidly in an emulsion of meconium in bile broth. The spore-bearing forms also increased in this medium. (b) A Gram-positive bacillus with rounded ends but shorter and more slender than (a), and frequently showing beaded or irregular staining. Rarely one of these organisms had a densely staining knob at one extremity, but *no bifid forms were seen in the true meconium period*. They occurred separately for the most part, but occasionally two individuals were joined end to end (see Plate XIX. Fig. 1). In an emulsion made in bile broth, these bacilli multiplied readily, and numerous forms showed the knobbed extremity, which stained deeply by Gram's method. After longer incubation this effect was lost, and the knob or headlet remained clear and refractile, like a spore. (c) Gram-positive cocci, usually in pairs or tetrads. (d) Gram-negative bacilli. In the first twenty-four hours of life these bacilli are less numerous than the Gram-positive varieties, and cultures on suitable media at this period give a predominance of Gram-positive over Gram-negative varieties.

It was frequently observed that cultures plated from bile-broth emulsions at this stage gave only cocci and Gram-positive bacilli, and no Gram-negative bacilli were obtained at all on bile-albumin plates.

II. From Third to Seventh Day.

Smears from faeces at this period have lost the uniformity of the meconium picture. Bacteria are very abundant, and the variety of forms has increased greatly. The large Gram-positive organism of the meconium period may be seen in small numbers in direct smears, and in bile-broth emulsions multiplies and is easily recognised. The slender Gram-positive bacillus can also be detected, and is distinguished by its beaded staining and the occasional presence of the headlet at one end. Scattered bifid forms also stained

in a beaded manner can be found in the direct smear, but have never been observed in the bile-broth emulsion.

Cocci are very numerous, and appear in chains and masses. A Gram-negative coccus is very constantly present at this stage.

Gram-negative bacilli are now very numerous, and appear in a considerable variety of forms. None of them, however, have been shown to be obligate anaerobes on culture, and accordingly their classification will not be further considered.

Description of Organisms isolated in Pure Culture.

By plating and subculture under conditions of strict anaerobiosis, two varieties of bacilli have been obtained.

(a) One of these corresponds to the large Gram-positive organism observed constantly in meconium, and has the morphological and cultural characters of *B. aerogenes capsulatus* (*B. perfringens*; *B. velehi*).

This is a non-motile bacillus, which spores freely under natural conditions, but not in pure culture. Its morphology is constant in culture. Hiss' serum-water medium was used in Durham fermentation tubes to test its action on glucose, mannite, dulcitol, lactose, and cane sugar. In a Bulloch's apparatus the fermentation tests are complicated by the fact that the gas produced does not remain in the tube, so that interpretation of results is difficult. Glucose and lactose showed much acid and gas production with heavy precipitate in the serum-water medium. Saccharose was attacked by some strains. Mannite was bleached, but recovered its colour in the air. Dulcitol was not affected. No indol reaction in peptone broth after five days. No growth in gelatin at 20°. At 37° growth took place with abundant gas production.

In milk the typical "stormy fermentation" took place in twenty-four hours, followed by separation of the clear fluid of the upper layer from the tough gas-blown clot below.

No animal inoculations have yet been made. The presence of this organism in large numbers in the faeces has been held to be associated with pathological conditions in older infants.

Its constant occurrence in healthy infants during the first week of life is of great interest, but the facts do not point to its possessing any pathogenic activity at this stage.

(b) The second anaerobic organism was isolated after considerable difficulty in pure culture. It could be demonstrated in direct smears from the faeces in every case examined.

It is a non-motile slender bacillus with rounded ends and from 4 to 7 μ in length. It retains Gram's stain, but the staining element is irregularly distributed, giving it a beaded appearance. The beading is well shown in preparations stained by methylene-blue (see Plate XX. Fig. 6).

The first pure cultures were obtained in deep glucose agar by the lateral symbiotic stab method above described, and grew about 2 cms. below the surface. From these cultures growths were obtained on surface of sloped ox-blood agar in scattered scanty colonies which never coalesce and have

irregularly serrated edges. The vitality of these cultures becomes impaired with repeated subculturing, and growths are obtained with difficulty after six weeks in the laboratory. As with many other strict anaerobes, the reaction to Gram's stain varies with the age of the culture. A smear of a young pure culture is Gram-positive for the most part, but a few individuals fail to retain the stain, and others stain only in part of their length. If a culture is examined from day to day the loss of the Gram-staining property becomes very marked, and the irregular beaded appearance of the organisms that retain the stain, is very striking. Cultures in fluid media or in deep agar stabs lose the Gram-staining property much earlier than surface growths. It has already been stated that the knobbed end which is formed stains deeply blue with Gram's stain when it first appears, and later is clear and refractile and fails to stain at all. *It does not stain with carbol fuchsin in the method used for demonstrating spores.*

Morphology.—The most striking character of this organism is its polymorphism. Repeated transfers have been made from plates to surface media, fluid media, and deep stabs, to demonstrate the existence of only one variety of organism, as the diverse appearances in different media suggested contamination or mixed cultures. The same appearances, however, have been noted with different strains of the bacillus after elaborate precautions had proved no contamination of the pure culture. The accompanying photomicrographs show the variations of vigorous cultures on different media and best illustrate the marked pleomorphism.

PLATE XIX. FIG. 2.—*An early pure culture on albumin-bile plate.* It indicates the regular parallel arrangement seen at this stage (thirty-six hours' incubation). Many long threads are present, and great variations in length may be noticed. The whorl-like curved disposition of the parallel organisms is at times very striking.

PLATE XIX. FIG. 3.—*Pure culture on blood-agar slope (forty hours).* This shows the early formation of clubbed or knobbed ends. These appear first as a small club-shaped swelling which gradually becomes sharply marked off and more rounded. At this stage also there is evidence of the loss of parallel arrangement, greater diversity in length, and tendency to the loss of Gram-stain.

PLATE XIX. FIG. 4.—*Culture on blood-agar slope (twelve hours later).* There is a dense background of organisms which have wholly or partially lost the power to retain Gram's stain. Many long rods with "headlet" formation are present, and one rod shows a clear knobbed extremity. Several oval bodies in the field represent the knobbed ends detached from the rods.

PLATE XX. FIG. 5.—*Older culture on albumin-bile plate.* A similar condition to Fig. 4, but showing well the beaded appearance of the bacilli in cultures of two or three days' incubation. One detached end is well seen.

In some cases a later stage showed almost complete disintegration of the bacillary forms with large clumps of the detached oval or rounded ends, which had all reached the clear refractile stage.

PLATE XX. FIG. 6.—*Growth on bile serum (thirty-six hours).* The organisms are stained with methylene-blue to show their well-marked beaded character when grown on this medium.

PLATE XX. FIG. 7.—*Young pure culture in fluid medium* (equal parts of bile and broth). In fluid media and in deep stabs the individual organisms show curved or twisted outlines, and have frequently a streptothrix-like appearance. Terminal and lateral branching is present but is not well marked, and does not closely resemble the bifid ends of bacilli seen in smears. The figure shows a club-shaped organism similar to those seen in surface growths, but they are very scanty in fluid or deep stab media.

PLATE XX. FIG. 8.—*Pure culture in deep glucose agar stab ($\times 1500$).* It is usual to see curved, twisted, and elongated forms in this medium, and the

Gram's stain is rapidly lost. In the figure the pale forms are counterstained by carbol fuchsin. The formation of the "headlet" ends is rarely seen in deep stabs or in fluid media.

The investigation of the bio-chemical properties of this organism has been disappointing, and results are meagre, owing to the extreme difficulty of obtaining growth in pure culture in special media. A notable feature is the effect of bile in aiding its growth, as shown by comparing glucose agar stabs with and without the addition of 25 per cent. bile. In the bile and agar tubes good growth was present twenty-four hours before any colonies were visible in ordinary glucose agar.

In bile broth very marked reduction of bile pigment takes place with formation of bilirubin from biliverdin. The yellow colour of the altered fluid remains unchanged in the air, unlike the yellow colour of early nursing faeces, which is changed to green when exposed to the atmosphere.

Results of milk cultures have not been uniform, as the organism only survived in 30 per cent. of the milk flasks inoculated. In some of these, the odour of butyric acid could be detected; the acidity of the medium increased slightly from day to day, and a soft clot was formed in seven to nine days. In other flasks the organism died out in four to five days and no clot was formed.

Examination for fermentation of sugars is difficult and unsatisfactory, as so many of the subcultures fail to grow. Peptone water proved a poor medium. Hiss' serum water medium was therefore used with glucose, mannitol, dulcitol, lactose, and cane sugar. Mannitol was affected by all the strains examined, and was the first sugar to show results. Bleaching took place in the anaerobic chamber in inoculated mannitol fermentation tubes (Durham's tubes), but control tubes were unaltered. The colour was restored in the air. Saccharose tubes showed slight acid production, and glucose tubes became definitely acid. A small amount of gas production is generally observed in deep glucose stabs, but when fermentation tubes are used in the anaerobic chamber, any gas formed is absorbed from the fluid, and accordingly results are inconclusive. Also it was necessary to test all fermentation tubes for living organisms, to confirm the results obtained, and subcultures were very often sterile. Negative results were therefore to be interpreted as a failure to obtain any culture at all.

Ordinary broth and glucose broth were unfavourable media, and therefore could not be used for testing indol production. No indol was found in bile broth after seven days.

In bile broth acidified with 1 per cent. acetic acid the organism grows fairly well, and survives longer than coliform organisms introduced with it. In this respect it resembles the members of the acidophilic group of the faecal flora.

There was no growth in gelatin at room temperature, but at 37° C. a fine granular sediment settled in the fluid gelatin.

On the whole the organism has the properties of a feeble fermenter of sugars, and is acidophilic, or "aciduric," as the term is used by Kendall.

The lengthy and detailed account of this organism is only justified by the fact that it is constantly present in the first days of life, and therefore indicates the early infection of the alimentary tract by anaerobic bacteria. It is important to know whether it persists in the faecal flora and whether it can be identified with Tissier's bacillus, and work is now proceeding on these lines.

The observations of Herter suggest that it is identical with Tissier's bacillus, but in the present investigation it is found to differ in the lack of bifurcations in cultures, in its failure to grow in glucose broth, and in its capacity for surface growth, which Tissier failed to obtain.

It is apparently identical with Rodella's organism *No. III.*, but shows more varied morphology than that observer described.

According to Herter, the organism known as Rodella's *B. III.*, is the early representative of the form which at a later period is the dominating organism of the intestinal flora of the healthy nursing, and is known as *B. bifidus*.

SUMMARY.

1. The addition of ox-bile in large quantities to the media favours growth of strict anaerobes in culture of early intestinal contents.
2. Two types of strict anaerobes appear in the intestinal tract eight to twelve hours after birth, and in the first forty-eight hours of life are more abundant than the facultative anaerobes of the coliform type.
3. The powerfully fermentative and proteolytic *B. aerogenes* was present within twelve to twenty-four hours after birth, in all cases examined. The other anaerobe, also constantly present, is acidophilic and a feeble fermenter of sugars, is markedly pleomorphic, and is affected by symbiosis and by variations in culture media.
4. At the end of the first week of life, facultative anaerobes of the coliform type are numerous, but the same two obligate anaerobes are still present in fairly large numbers, and can be demonstrated by cultivation in special media.

My acknowledgments are due to Dr. D. Reid for preparing the micro-photographs.

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DESCRIPTION OF PLATES XIX.-XX.

PLATE XIX.

- FIG. 1.—Smear from meconium, two days after birth. Gram's stain. ($\times 1000$.)
 FIG. 2.—Colony from bile-albumin plate. Gram's stain. ($\times 1000$.)
 FIG. 3.—Colony on blood-agar slope. Gram's stain, carbol-fuchsin counterstain.
 ($\times 1000$.)
 FIG. 4.—Later stage of colony on blood-agar slope. Gram's stain, carbol-fuchsin
 counter-stain.

PLATE XX.

- FIG. 5.—Older culture on bile-albumin plate. Gram's and carbol-fuchsin stain. ($\times 1000$.)
 FIG. 6.—Colony from bile-serum plate. Methylene-blue stain. ($\times 1000$.)
 FIG. 7.—Young pure culture in bile broth. Gram's and carbol-fuchsin stain. ($\times 1000$.)
 FIG. 8.—Pure culture in deep glucose agar stab. Gram's and carbol-fuchsin stain. ($\times 1500$.)



FIG. 1.

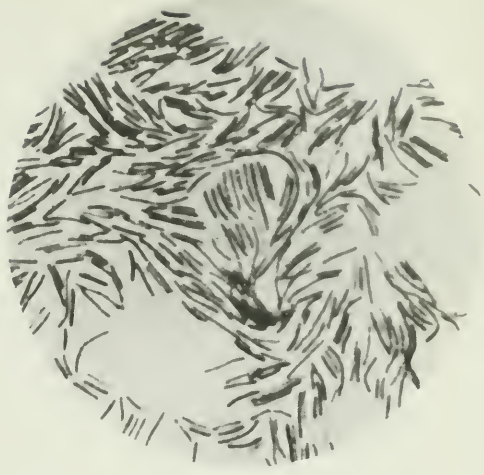


FIG. 2.



FIG. 3.

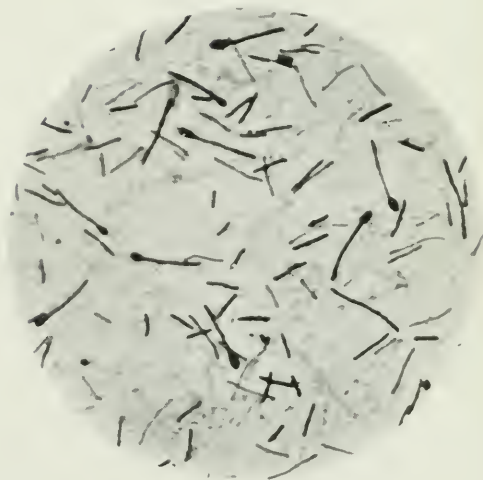


FIG. 4.



FIG. 5.

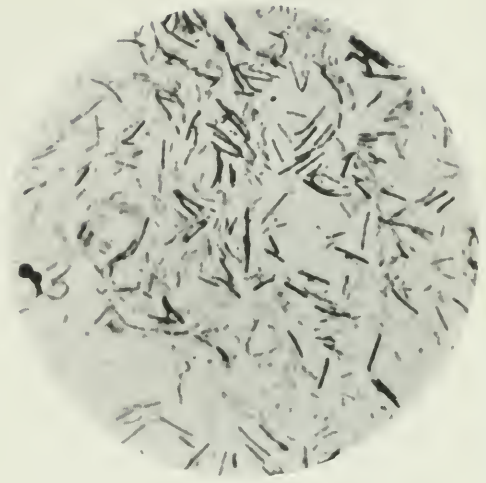


FIG. 6.



FIG. 7.

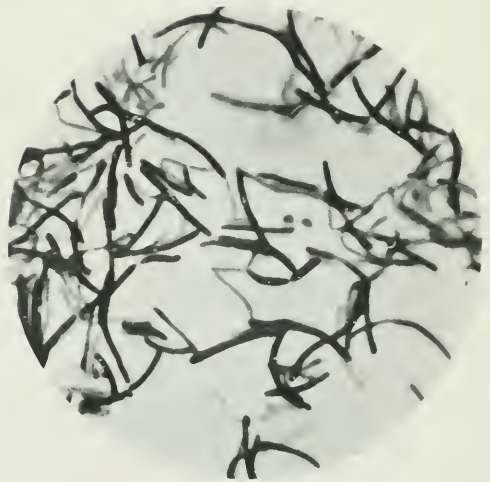


FIG. 8.

THE INFLUENCE OF THE AGE OF THE PARENT ON THE VITALITY OF THE CHILD—A FIRST STUDY.

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THE subject I propose to discuss in the present and subsequent papers is of importance in two ways. Popular traditions have frequently implied that the age at which a parent begets offspring is not without influence upon the characters of the latter, the prevailing belief being humorously portrayed in the late W. S. Gilbert's ballad "The Precocious Baby." Again, recent thinkers, notably Professor Karl Pearson, have asserted that elder born children differ markedly in liability to certain diseases from their younger brethren. Were this view to be accepted it would be relevant to enquire whether the difference might not be a function of the age of the parents at the time of birth of the offspring or at least whether such difference might not play a part in bringing about this result. Assuming that any such effect could be demonstrated, it is plain that a valuable stimulus would be given to the study of physiological changes in the reproductive system within the fertile period. These are the immediately interesting aspects of the subject, but with them I am less directly concerned than with certain secondary consequences.

To state that an essential preliminary of a comparison is that the things to be compared must be in *pari materia*, may seem unnecessary, but this truism is in constant danger of being forgotten. We are all aware that the crude birth or death rates of different communities cannot be made the subjects of a valid comparison, but that corrections for age and sex distribution are necessary. The introduction of these

corrections has, however, tended to engender a feeling of undue confidence in the ease of interpreting such adjusted ratios. Quite recently warnings have emanated from various quarters with regard to this point. Thus Brownlee has pointed out in this *Journal* that the customary employment of a corrected death rate as a measure of salubrity is not free from doubt, while Hamer in his presidential address to the epidemiological section of the Royal Society of Medicine and again in his annual report to the London County Council has dwelt upon the possible importance of migration as a factor in death and birth rates which cannot be entirely allowed for by means of age and sex corrections. Evidently if the age at which a child is begotten or conceived, influences not merely the probable number of its brothers and sisters but also its own physical or mental characters, we have to reckon with another factor in the interpretation of a changing death or disease rate. It will not be sufficient to allow for the age constitution of a community, it will be necessary to consider that of the previous generation as well, since, whatever else may be obscure, it is certain that a small but significant increase in the average age at marriage has taken place in most sections of the community. For these reasons, it is plain that an attempt to measure the influence of the ages of parents at the time of procreation or conception upon the longevity of their offspring is worth making, and this paper contains an account of a preliminary effort. I shall not try to summarise the literature, at once voluminous and meagre, which has some more or less direct bearing upon my theme; much of it is merely traditional and theoretic, little of it is based upon exact statistical data handled in an appropriate fashion. Stanley Hall's well-known book on adolescence contains references to various earlier papers, and additional citations will be found in Gini's 1912 paper, while among writers who have treated of the influence of order of birth, mention should be made of Lucien March, Karl Pearson and his associates, Weinberg, Yule and Greenwood, Goring and Ploetz. I may also refer to some preliminary notes of my own, cited in the list of references appended. This paper is confined to a study of the influence of parental age upon the longevity of offspring and age at marriage, and the data used will in the first place need careful description. These data fall into four series, viz. :

The first was collected from Burke's *Peerage*, 1902, and related to the possible influence of the age of the father at birth, on the age at death of sons who had survived to the 15th year; the first male born of the first male in each generation only being considered.

The second was collected in Middlesbrough, through the agency of the Medical Inspection of School Children, and deals with the age of the grandmother at the birth of the mother and the number of children dying before adult life.

The third also collected in Middlesbrough, through the agency of the Notification of Births Act, permitted the consideration of the age of mother at birth of the offspring and its chance of living one year.

The fourth and last collected through the same agency as the third, had for its object the possible association between age and the chance of foetal death prior to term.

It will be observed, that, though there is some overlapping, all periods of life are covered and further that both parents have been dealt with. Unfortunately data were not obtainable in the *Peerage* with respect to the age of the mother. It is obvious that in an enquiry of this kind, both parents ought to be considered, for should an association be found to exist in the case of the male parent, it might arise from the ages of the uniting ovum and sperm or the environmental influence of the mother's age, subsequent to fertilization. We can now consider each series in detail, indicating the corrections that should be made and such fallacies as are dependent upon the method of collecting the data.

Age of Father at Son's Birth, and Age at Death of Adult Sons.

The peculiarities of this series of observations which should be borne in mind are as follows:

(a) The oldest males only were considered. That is, only instances where the father was the eldest born and the son whose age at death was recorded was also the first male born. Unfortunately the actual position in the family of the person under consideration is not given in the raw material.

(b) Only such sons as survived the 15th year are considered.

(c) All deaths through violence were excluded so far as recorded.

It has already been shown by Beeton, Pearson and Yule, that a correlation exists between the length of life of the father and that of the son which is equal to $\cdot 12$. Now it is obvious that a child born of an elderly parent may be influenced in two ways: (1) He may tend to survive to a mature age because his father has already done so and (2) his life expectancy may be curtailed because his parents were

old when he was born. Hence partial correlations must be formed, with the age of the father at death constant. The following three correlation tables were drawn up:

- (I) Age of father at birth of son and age of son at death.
 (II) Age of father at death and age of son at death.
 (III) Age of father at birth of son and age of father at death.

TABLE I.
*Age of father at death and age of son at death. First male births and such
 as survive to adult life.*

Age of Father		Age of Son																								Totals																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																								
29-32	33-36	37-40	41-44	45-48	49-52	53-56	57-60	61-64	65-68	69-72	73-76	77-80	81-84	85-88	89-92	93-96	97-100	101-104	105-108	Totals	2	8	22	43	40	49	55	63	84	90	78	67	46	13	4	1	2	1	795																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																											

$\sigma_x = 3.8431$. $\sigma_y = 3.8933$. $r = .2248 \pm .0248$.

TABLE III.

Age of father at birth of eldest son and death of father.

		Age at death																																									Totals			
Age at birth		30	32	34	36	38	40	42	44	46	48	50	52	54	56	58	60	62	64	66	68	70	72	74	76	78	80	82	84	86	88	90	92	94	96	98	100	102	104	106	108	110	116			
20	1																																											5		
22	1																																											43		
24	2																																											78		
26																																												104		
28	2																																											135		
30	2																																											126		
32																																												140		
34	2																																											1		
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Totals	5	3	4	10	11	27	11	15	33	43	42	38	27	48	41	41	59	49	58	70	69	61	70	85	67	43	40	49	33	23	7	11	4				1		1	1	1201					

$\sigma_x = 4.1604, \sigma_y = 7.3045, r = +.2543 \pm .0229.$

 $\sigma_x = 4.1604, \sigma_y = 7.3045, r = +.2543 \pm .0229.$

The values of the correlation disclosed by the tables are as follows :

Age of father at birth (1) and son at death (2), $r = .0345 \pm .023$

Age of father at death (3) and son at death (2), $r = .2248 \pm .0248$

Age of father at birth of son (1) and death (3), $r = .2543 \pm .0229$

It is to be observed that the second coefficient is much larger than that previously given by Prof. Pearson and Miss Beeton, namely $.135 \pm .0209$, and the matter needs further discussion. In the first place the correlation surface is not a random sample of the type descriptive of the degree of association obtaining in the whole "population" of fathers and sons owing to the restriction as to age and birth order already mentioned. So far as the former is concerned the same selection was practised by Beeton and Pearson in their paper of 1899 (*Proceedings, Royal Society*) which also dealt with peerage data, and the correlations they obtained are decidedly smaller than mine; but they did not restrict the analysis to first male births. This may partly account for the discrepancy between our results. It will be noticed that there is far less difference in mean age and variability of father and sons in my data than in that of Beeton and Pearson. It will be remembered that the latter pointed out (*op. cit.* p. 292, etc.) that the filial generation was less stringently selected than the parental. The observed correlation was necessarily lowered, there being a mixture of material due to the operation of that fraction of the death rate which is really non-selective upon the sons. It appears probable that the further selection in confining oneself not only to adult firstborn males, but to those who actually survived their fathers, is a sufficient explanation of the difference. Whether my value is a really adequate measure of the force of inheritance in respect of longevity and what part is played in heredity by the influence of the age of the parents at birth of the son cannot be stated off-hand.

In the following table (p. 460) are the constants as given by Beeton and Pearson in their 1899 paper and found from the above data.

If we now make age of father at death constant then

$$r_{12} = -.0431 \pm .0239.$$

The conclusion or rather suggestion is that as the father increases in age at the birth of the son, the life expectancy of the son, who has already survived to the 16th year, tends to be curtailed. The correlation ratio for means of arrays of sons' ages at death for various ages of fathers at birth of sons $= .3394 \pm .0389$, and when corrected by Pearson's method (*Biometrika*, VIII) the value approximates to $.3042$.

	Beeton and Pearson's data (1899)	Present Paper
Mean age of Fathers at death* ..	65·835	67·14
Mean age of Sons at death ..	58·775	65·84
Standard Deviation (Fathers) ..	14·6382	14·24
Standard Deviation (Sons) ..	17·0872	14·20
Coefficient of correlation	0·1149 ± ·0210	·2214 ± ·0248
Correlation ratio (Fathers) † ..	—	·3394 ± ·0389
Corrected ‡	—	·3042

* The mean age of fathers who were Peers is one and a half years greater than the sons who succeeded them. This is due to the fact that a proportion did not inherit the title and hence would be of a mature age when it was conferred upon them.

† Any distribution has two correlation ratios; the word in brackets indicates which has been calculated, *e.g.* correlation ratio (fathers) denotes the coefficient obtained from the means of arrays of fathers.

‡ The correlation for η is calculated by the method described by Prof. Pearson, 1912.

If we replaced number of years lived by the death rate at ages and thus reversed the order of our categories, the association would become positive and we might say that the death rate at ages increases with the age of the father at birth. This change as will be seen subsequently would bring the present series of observations into line with the remainder.

The Age of the Grandmother at the Birth of the Mother and the Number of Children dying in the Family.

This enquiry as already stated was carried out in Middlesbrough through the agency of the Education Act (Administrative Provisions) 1907, or what is known as the Medical Inspection of School Children. The method was as follows: All children in their 8th year were selected and their parents invited to attend the examination; of the total number, 50 % complied. The sample thus obtained can be regarded as comprehensive and is such that it reaches each family once only. It is obvious, however, that the material is a selection and not a random sample because it ignores all sterile matings and those in which all the children born have died before the 8th year; further the representation of families of various sizes is directly proportional to the number that survive. Hence the larger the family the greater the chance of its possessing a member 8 years old.

According to the work of Weinberg, Macaulay, Yule and Greenwood it appears that considerable caution must be exercised in basing deductions on data collected in this way, as the possible statistical fallacies are numerous and in some respects cannot be corrected.

One error which tells against a positive conclusion arises from the fact that if age at birth is detrimental to the survival value of the family, it is obvious that those families which have been hit the hardest are only represented in our data in proportion to the chance of one of the survivors being 8 years old, which, even if the family was originally large, might be small, under the method of selection adopted.

It is to be regretted that no allowance has been made for the length of time the mother had been married, as the age at marriage was not obtained in the original data. To assume that the mean reproductive periods in the categories chosen will be approximately equal is an assumption that is hardly justifiable. Bearing these reservations in mind we may proceed to discuss the actual findings. The information obtained from each mother or responsible guardian was (1) present age of mother; (2) age of grandmother if living, or age she would have been had she lived; (3) the number of children born to mothers—still births are included, but not miscarriages; (4) the number dead.

It is to be noted that information relative to ages is fairly reliable, owing to the widespread use of insurance against burial expenses at death. As in the previous case, partial correlation was used and the problem was to find the value of the association, if any, between the age at which the mother was born and the number dying for total number born constant.

The correlations for the above tables are as follows:

Age of grandmother at birth of mother (1) and number dead (2):

$$r_{12} = \cdot 0238 \pm \cdot 0213.$$

Number of children dead (2) and size of family (3):

$$r_{23} = \cdot 7230 \pm \cdot 0109.$$

Size of family born (3) and age of grandmother at birth of mother (1):

$$r_{13} = \cdot 0641 \pm \cdot 0150.$$

Testing our crude correlations for linearity, we have for age of grandmother at birth of mother and number dying:

$$\eta_{12} \text{ (number dead)} = \cdot 076.$$

Corrected $\eta_{12} = \cdot 0314 \pm \cdot 0209,$

$$\eta_{13} \text{ (age)} = \cdot 139.$$

Corrected $\eta_{13} = \cdot 1015 \pm \cdot 0208.$

There may be some non-linearity with respect to the age categories, still a partial correlation should give a little information.

TABLE IV.

Age of grandmother at birth of mother and number of children dead.

Age at birth	0	1	2	3	4	5	6	7	8	9	10	11	12	No. dying, Means
20 yrs. and under ..	38	24	30	6	6	5	4	1	1		1			116
21st to 25th year ..	95	68	45	23	19	5	3	2	1		1			1-724
26th to 30th year ..	95	75	49	25	11	6	3	2	1	2	2	1	1	1-528
31st to 35th year ..	78	46	42	15	16	6	5	4	2		1	1		273
36th to 40th year ..	31	32	19	15	8	3	1	3	1					1-741
41st and over	22	13	11	7	1	3	1	1						1-743
Totals	359	258	196	91	61	28	17	13	6	2	5	2	1	59
Means	3-09	3-12	3-04	3-34	3-06	3-21	3-52	3-69						1039

 $\sigma_x = 1-351$. $\sigma_y = 1-8486$. $r = -0-238 \pm -0-213$.

TABLE V.

Age of grandmother at birth of mother and number of children born to mother.

Age at birth	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	No. of children born
20 yrs. and under ..	6	16	19	14	22	15	11	9	4	3	5	1	1	3	1		129
21st to 25th year ..	14	28	65	57	43	28	17	21	26	9	4	1	3	3		1	320
26th to 30th year ..	17	49	50	43	53	34	17	16	14	10	5	3	2	5			318
31st to 35th year ..	17	42	46	45	48	24	13	14	8	7	4	3	1	1	2		273
36th to 40th year ..	15	25	40	18	16	11	10	10	6	3	4	1	1				162
41st and over ..	1	17	19	16	4	9	7	7	2	2	3	1	2	1			91
Totals ..	70	177	239	193	186	121	75	77	60	34	25	10	9	13	3	1	1293
Means ..	3-34	3-47	3-33	3-23	3-02	3-15	3-20	3-21	2-9	3-12	3-28	3-5	3-54	2-61	3-68	2-8	

 $\sigma_x = 1-473$. $\sigma_y = 2-8561$. $r = -0-064 \pm -0-15$.

TABLE VI.

Size of family and number dead.

No. dead	Size of family																			Totals
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	
0	31	73	71	82	49	27	16	7	4	4	1									365
1		12	44	51	62	36	15	21	14	4	0	1	1							261
2			14	20	27	38	30	40	11	11	3	1	1	1						197
3				1	10	17	13	18	17	10	7	2	0	2						97
4					1	6	6	10	10	13	8	1	1	3	2					61
5							5	2	1	1	4	6	5	2						26
6							1	3	2	3	1	4	1	1		1				17
7									2	1	2	2	2	1	1					11
8										1	0	1	1	1	1				1	6
9											1	0	0	1						2
10														3		1				4
11														1						1
12														1					1	2

Totals .. 31 85 129 154 149 124 86 101 61 48 27 18 12 17 4 2 0 0 2 1050
 $\sigma_x = 3.122$. $\sigma_y = 1.84$. $r = .723 \pm .0109$. Mean size of family 5.77. Mean number dead 1.56

Age of grandmother at birth of mother and number dying in the family, total number constant:

$$r_{12} = .0943 \pm .0174.$$

This would suggest that as the age at which the mother was born increases, the chance of her rearing all her children decreases. It is of interest to note that the association having regard to its probable error is more significant than in our first series. Had the present data been as reliable we should have been justified in assuming that the effect of parental age at birth is more marked in early life than at the more mature ages.

*The Age of the Mother at Birth of Child and its
 chance of living one year.*

The third series of observations dealing with the possible association between age of mother at birth and chance of living one year is also subject to certain reservations:

- (1) The data deal only with a poor class population.
- (2) Only such as remain in the town for one year are considered.
- (3) A fair number are lost owing to migration. These are usually the latter born, for it is the parents of such who are most affected by

trade fluctuations. The number lost in this way is a little over 5% of the whole number. The information collected was :

- (1) Age of mother at birth.
- (2) Order of birth. (Miscarriages and still births are included.)
- (3) Whether living or dead at end of the year. This information is of course furnished by the Registrar to all Health Departments. The method adopted was as follows: A table was formed (Table VII) with age of mother at birth and order of birth as abscissae and ordinates respectively, and in each appropriate square the number born and the number dead at the end of the year were placed and the death rate for each age and order worked out. This table was then turned into two sub-tables :

- (1) Infantile Mortality and age of mother at birth.
- (2) Order of birth and Infantile Mortality.

The figures in the squares being the number of observations upon which the various mortality categories are based. The third table, Age of mother at birth and order of birth, was taken from the original table.

The constants obtained from these tables are :

Age of mother at birth of offspring (1) and order of birth (3) :

$$r_{13} = \cdot 6128 \pm \cdot 0051.$$

Age of mother at birth of offspring (1) and infantile mortality (2) :

$$r_{12} = \cdot 1157 \pm \cdot 0142.$$

Order of birth (3) and infantile mortality (2) :

$$r_{23} = + \cdot 0367 \pm \cdot 0208.$$

Taking the age of mother at birth of offspring and infantile mortality, with order constant :

$${}_3r_{12} = \cdot 1179 \pm \cdot 0143,$$

and birth sequence and infantile mortality with age of mother at birth constant :

$${}_1r_{23} = - \cdot 0531 \pm \cdot 0205.$$

Testing these for linearity of regression, order of birth and infantile mortality

$$\eta \text{ (order)} = \cdot 2403, \eta \text{ (corrected)} = \cdot 2254 \pm \cdot 0145.$$

Age of mother and infantile mortality :

$$\eta \text{ (mortality)} = \cdot 2939, \eta \text{ (corrected)} = \cdot 2810.$$

TABLE VII. *Number of births and deaths in first year according to age of mother at birth and order.*

Years	1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th	11th	12th	13th	14th	15th	16th	17th	No. born	Deaths	Rate per 1000
	1																	1	—	0
15	—																	2	—	0
16	—																	7	2	286
17	2																	20	2	100
18	2	2	1															49	14	286
19	35	14																75	10	133
20	5	9																61	11	2
21	7	2	1	—														49	20	6
22	10	3	—	—	2													10	3	—
23	45	29	14	6														45	29	14
24	7	4	4	—														39	41	28
25	6	7	4	—	5													6	7	4
26	29	31	22	11	9	3	1											29	31	22
27	1	4	3	—	1	—	—											1	4	3
28	45	27	36	26	7	4	—											45	27	36
29	6	4	5	1	1	—												6	4	5
30	29	37	29	23	11	4												29	37	29
31	7	4	2	4	1	—												7	4	2
32	17	18	19	15	15	10	1	1	1									17	18	19
33	2	3	—	2	4	2	—	—	1									2	3	—
34	16	23	27	20	18	4	6	3										16	23	27
35	2	5	1	3	1	—	—	2	1									2	5	1
36	15	10	23	19	16	16	9	2	1									15	10	23
37	4	—	5	2	—	—	2	2	—									4	—	5
38	4	13	12	20	23	18	10	6	—									4	13	12
39	2	2	1	3	1	1	1	2	1									2	2	1
40	2	8	10	8	10	13	18	5	1									2	8	10
41	1	—	2	1	2	—	4	2	—									1	—	2
42	3	8	8	14	16	24	11	13	5									3	8	8
43	—	—	—	2	1	2	5	1	1									—	—	—
44	6	3	4	7	8	21	20	9	4									6	3	4
45	2	—	—	2	1	5	3	1	1									2	—	—
46	3	4	6	8	11	18	17	5	8									3	4	6
47	—	2	—	2	—	1	2	—	2									—	2	—
48	4	3	5	10	12	11	17	6	9									4	3	5
49	—	2	—	1	1	—	2	—	2									—	2	—
50	—	2	—	1	1	—	2	3	1									—	2	—
51	1	3	6	3	8	12	5	12	10									1	3	6
52	—	2	—	—	1	2	—	—	1									—	2	—
53	1	2	—	—	1	2	—	—	1									1	2	—
54	—	2	—	—	1	2	—	—	1									—	2	—
55	—	2	—	—	1	2	—	—	1									—	2	—
56	1	3	6	3	8	12	5	12	10									1	3	6
57	—	2	—	—	1	2	—	—	1									—	2	—
58	1	2	—	—	1	2	—	—	1									1	2	—
59	—	2	—	—	1	2	—	—	1									—	2	—
60	—	2	—	—	1	2	—	—	1									—	2	—
61	—	2	—	—	1	2	—	—	1									—	2	—
62	—	2	—	—	1	2	—	—	1									—	2	—
63	—	2	—	—	1	2	—	—	1									—	2	—
64	—	2	—	—	1	2	—	—	1									—	2	—
65	—	2	—	—	1	2	—	—	1									—	2	—
66	—	2	—	—	1	2	—	—	1									—	2	—
67	—	2	—	—	1	2	—	—	1									—	2	—
68	—	2	—	—	1	2	—	—	1									—	2	—
69	—	2	—	—	1	2	—	—	1									—	2	—
70	—	2	—	—	1	2	—	—	1									—	2	—
71	—	2	—	—	1	2	—	—	1									—	2	—
72	—	2	—	—	1	2	—	—	1									—	2	—
73	—	2	—	—	1	2	—	—	1									—	2	—
74	—	2	—	—	1	2	—	—	1									—	2	—
75	—	2	—	—	1	2	—	—	1									—	2	—
76	—	2	—	—	1	2	—	—	1									—	2	—
77	—	2	—	—	1	2	—	—	1									—	2	—
78	—	2	—	—	1	2	—	—	1									—	2	—
79	—	2	—	—	1	2	—	—	1									—	2	—
80	—	2	—	—	1	2	—	—	1									—	2	—
81	—	2	—	—	1	2	—	—	1									—	2	—
82	—	2	—	—	1	2	—	—	1									—	2	—
83	—	2	—	—	1	2	—	—	1									—	2	—
84	—	2	—	—	1	2	—	—	1									—	2	—
85	—	2	—	—	1	2	—	—	1									—	2	—
86	—	2	—	—	1	2	—	—	1									—	2	—
87	—	2	—	—	1	2	—	—	1									—	2	—
88	—	2	—	—	1	2	—	—	1									—	2	—
89	—	2	—	—	1	2	—	—	1									—	2	—
90	—	2	—	—	1	2	—	—	1									—	2	—
91	—	2	—	—	1	2	—	—	1									—	2	—
92	—	2	—	—	1	2	—	—	1									—	2	—
93	—	2	—	—	1	2	—	—	1									—	2	—
94	—	2	—	—	1	2	—	—	1									—	2	—
95	—	2	—	—	1	2	—	—	1									—	2	—
96	—	2	—	—	1	2	—	—	1									—	2	—
97	—	2	—	—	1	2	—	—	1									—	2	—
98	—	2	—	—	1	2	—	—	1									—	2	—
99	—	2	—	—	1	2	—	—	1									—	2	—
100	—	2	—	—	1	2	—	—	1									—	2	—

The regression, therefore, is markedly non-planar. This is probably due to the fact that the data are heaped up in the two extreme categories, namely, mortality of $\cdot 0\text{--}5\%$, the largest portion consisting of families in which none have died in the first year and mortalities of $90\text{--}100\%$, mainly those families in which all have died (a fact which in the majority of cases was due to syphilis). In spite of these reservations, I think we can conclude that as the age of the mother advances, the chance of the child surviving decreases, and as the family gets larger, other things being constant, the probability of its living one year increases. Findings which are on the whole corroborated by one's general impressions. Since the third series is restricted to infantile mortality which is only a part in the second series, the suggestion is that the influence of parental age hardly affects the adolescent period of life. It must, however, be remembered that the methods of tabulation were not the same in the two cases.

Present Age of Mother and Number of Accidents in previous Family.

The fourth and last series was obtained with the object of ascertaining the possible association between the age of the mother and the death of the foetus prior to full gestation; the data were collected in Middlesbrough on the occasion of a birth. The mother being asked (1) the number of live births she had had; (2) the number of deaths in the first year; (3) the number of miscarriages and still births. It is to be noted first, that the enquiry was limited to certain working class districts of the town, though all births were reached that occurred in that area, and secondly, that each category is assumed to include the experience of the previous ones. This is not quite true, as the smaller families tend to be restricted to ages centring round the 29th year and are more rarely found in the early and late periods. The data are on these accounts obviously not homogeneous. The findings, however, in the previous series that size of family does not seriously affect infantile mortality when age is made constant, should remove this objection.

The data are as follows:

TABLE VIII.

*Histories of mothers with respect to accidents, deaths in first year,
and number of births in first year.*

Age of mother	No. of enquiries	No. of births	Accidents	Rate	No. of deaths	Rate
16	4	4	0	0	0	0
17	10	10	0	0	0	0
18	26	32	6	188	2	63
19	51	63	1	16	6	95
20	82	111	3	27	9	81
21	89	154	12	78	14	26
22	102	218	19	87	16	73
23	129	313	19	61	31	99
24	118	321	24	75	39	121
25	110	321	18	56	37	115
26	110	384	31	81	48	125
27	100	399	36	90	66	165
28	103	421	26	62	66	157
29	98	463	27	58	59	127
30	101	426	41	96	75	176
31	68	307	16	52	31	101
32	88	522	36	69	61	117
33	75	479	32	67	66	138
34	77	559	68	122	69	123
35	61	437	26	60	44	111
36	63	413	25	61	59	143
37	54	425	33	78	68	160
38	44	396	47	119	45	114
39	40	381	61	158	62	163
40	41	393	34	87	60	153
41	9	110	8	73	17	155
42	23	289	44	152	44	152
43	12	138	13	94	12	86
44	12	128	12	94	30	234
45	3	42	4	95	1	24
46	3	30	4	133	1	33
47	1	9	0	0	2	222
48	1	13	0	0	1	77
49	1	12	0	0	2	106
Totals	1909	8728	726	86	1143	119

Correlating these directly and using the column "Number of Enquiries" as weights, we have:

Present age of mother and infantile mortality in previous family:

$$r = .0425 \pm .0210;$$

present age of mother and accident rate in previous family :

$$r = \cdot 2886 \pm \cdot 018 ;$$

infantile mortality rate and accident rate :

$$r = \cdot 4006 \pm \cdot 0064.$$

Considering the problem from the point of view of individual families we see that as the age of the mother increases so does the chance of the child dying either before or after birth increase, the correlation in the former case being about seven times that in the latter. It is of interest also to note the high correlation between death before and after birth.

Putting the foregoing results together we have,

- (1) Present age of mother and miscarriage rate in previous family :

$$r = \cdot 2886 \pm \cdot 018 ;$$

- (2) Age of mother at birth of offspring and infant mortality :

$$r = \cdot 1179 \pm \cdot 0143 ;$$

- (3) Age of grandmother at birth of mother and number of family dying before adult life :

$$r = \cdot 0943 \pm \cdot 0174 ;$$

- (4) Age of father at birth of son and longevity of sons :

$$r = - \cdot 0431 \pm \cdot 0239.$$

If, however, we could exclude from (3) the association due to infantile mortality, it might approximate to zero or actually become negative. It is possible that we are dealing with two different things, namely, sthenic and asthenic deaths. In the former the reactions of the organism are so excessive as to cause death, whilst in the latter the death is due to their relative absence. In infancy and late life death is mainly asthenic, whilst in the adolescent period it is sthenic. If this is so, then a negative correlation for the adolescent period would be expected. The above consideration, though it goes beyond the data, is a useful suggestion as it indicates that the next step in the enquiry must be the consideration of age of parent at birth of offspring and cause of death, age at death being kept constant.

Age of Father at birth of Son and Age of Son at Marriage.

As evidence has already been given which lends considerable force to the belief that the number of years that a person will live partly depends upon the age of the parents, when he or she was born, it

is not unreasonable to consider whether fertility is also influenced by the same factor. Before such an enquiry can be undertaken, the factors limiting the reproductive period must be clearly defined. The termination, that is death, has already been dealt with in the present paper, and the beginning, namely age at marriage, will now be examined.

The data on which this investigation is based have been taken from Burke's *Peerage*, and to produce as much homogeneity as possible only eldest born sons of eldest born fathers are considered. The characters chosen were :

- (1) Age of father at birth of his son.
- (2) Age of son at marriage.
- (3) Age of father at death.
- (4) Age of father at marriage.

Beyond the two fundamental factors it is necessary to allow for any hereditary tendency, first in respect to marriage, for if the father mates early the son is likely to do so, and secondly in respect to death, for if the father dies early it is likely to hasten the marriage of the son, as he will assume his full authority at an earlier date, and should he be a minor at the time there will be every social reason for his marriage at the earliest moment that convention allows, hence it is necessary to examine any association that may exist between the factors: Age of the father at marriage and age of son at marriage, Age of father at death and age of son at marriage, and should such prove to exist, suitable allowance must be made. The values are as follows :

Age of father at birth of son (1) and age of son at marriage (2) :

$$r_{12} = -\cdot 0175 \pm \cdot 0247.$$

Age of father at birth of son (1) and age of father at marriage (4) :

$$r_{14} = \cdot 7338 \pm \cdot 0114.$$

Age of father at birth of son (1) and age of father at death (3) :

$$r_{13} = \cdot 2534 \pm \cdot 0232.$$

Age of father at marriage (4) and age of father at death (3) :

$$r_{34} = \cdot 1620 \pm \cdot 0239.$$

Age of son at marriage (2) and age of father at death (3) :

$$r_{23} = \cdot 2219 \pm \cdot 0236.$$

Age of son at marriage (2) and age of father at marriage (4) :

$$r_{24} = \cdot 0463 \pm \cdot 0243.$$

TABLE IX.
Age of father at birth of son and age of son at marriage.

Age of father at birth		Age of son at marriage																				Totals										
		15-16	17-18	19-20	21-22	23-24	25-26	27-28	29-30	31-32	33-34	35-36	37-38	39-40	41-42	43-44	45-46	47-48	49-50	51-52	53-54	55-56	57-58	59-60	61-62	63-64	65-66	69-70	71-72	77-78		
15-16																															3	
17-18																															4	
19-20																															26	
21-22																															118	
23-24																															130	
25-26																															137	
27-28																															110	
29-30																															88	
31-32																															64	
33-34																															63	
35-36																															43	
37-38																															35	
39-40																															21	
41-42																															16	
43-44																															7	
45-46																															5	
47-48																															4	
49-50																															3	
51-52																															2	
53-54																															1	
55-56																															2	
57-58																															1	
59-60																															2	
61-62																															1	
63-64																															1	
65-66																															1	
67-68																															1	
73-74																															1	
Totals		2	3	2	30	71	84	107	93	99	77	73	66	51	42	24	17	15	11	15	6	5	5	5	3	1	3	2	1	1	1	910

$\sigma_{\text{birth}} = 3.7673$. $\sigma_{\text{marriage}} = 4.3389$. $r = -.0175 \pm .0247$.

$\sigma_{\text{birth}} = 3.7673$. $\sigma_{\text{marriage}} = 4.3389$. $r = -.0175 \pm .0247$.

TABLE X.

Age of father at marriage and age at birth of eldest son.

Birth of eldest son		Age at marriage																								Totals
		13-14	15-16	17-18	19-20	21-22	23-24	25-26	27-28	29-30	31-32	33-34	35-36	37-38	39-40	41-42	43-44	45-46	47-48	49-50	51-52	53-54	55-56	57-58	59-60	
15-16																										
17-18			2																							
19-20			1	18																						
21-22			1	3	12	17	13	22																		
23-24		1			37	24	40	19																		
25-26					36	11	22	11																		
27-28					8	6	11	10	19																	
29-30			1	2	5	11	11	8	34	11																
31-32				1	8	6	11	10	7	13	9															
33-34				1	2	2	3	3	8	8	18															
35-36					2	1	2	2	2	6	6															
37-38					1	1	3	1	2	3	4	9														
39-40					3	5		2	5	3	5	8														
41-42					1	2	2	1	1	2	4	14														
43-44							2	2	1	1	1	5														
45-46				1			2	1	1	2	1	2						2								
47-48							1	1		1	1	1						3	1							
49-50									1		1	1								1						
51-52										1										3						
53-54							1																			
55-56					1																					
57-58																										
59-60																										
61-62																										
63-64																										
65-66																										
67-68																										
69-70				1				1																		
71-72										1																
Totals	1	3	8	30	115	104	99	92	76	47	44	35	31	17	13	10	5	2	5	2	2	1	5	1	746	

$\sigma_{\text{age at birth}} = 4.3112, \quad \sigma_{\text{marriage}} = 3.5564, \quad r = .7338 \pm .0114.$

 $\sigma_{\text{age at birth}} = 4.3112, \quad \sigma_{\text{marriage}} = 3.5564, \quad r = .7338 \pm .0114.$

Vitality of the Child

TABLE XI.
Age of father at marriage and age of father at death.

Age at death	Age at marriage																			Totals
	13-14	15-16	17-18	19-20	21-22	23-24	25-26	27-28	29-30	31-32	33-34	35-36	37-38	39-40	41-42	43-44	45-46	47-48	49-50	
23-25																				1
26-28																				4
29-31																				8
32-34																				21
35-37																				11
38-40																				39
41-43																				38
44-46																				45
47-49																				52
50-52																				60
53-55																				80
56-58																				87
59-61																				98
62-64																				74
65-67																				66
68-70																				46
71-73																				13
74-76																				5
77-79																				1
80-82																				1
83-85																				1
86-88																				1
89-91																				1
92-94																				1
95-97																				1
98-100																				1
101-103																				1
104-106																				1
Totals	1	3	8	30	117	104	99	92	75	47	44	35	32	18	13	10	5	2	6	750

$\sigma_{\text{death}} = 3.5118$, $\sigma_{\text{marriage}} = 3.4230$, $r = .16198 \pm .0239$.

TABLE XII.

Age of son at marriage and age of father at death.

Age at death	Age at marriage																								Totals	
	13-14	15-16	17-18	19-20	21-22	23-24	25-26	27-28	29-30	31-32	33-34	35-36	37-38	39-40	41-42	43-44	45-46	47-48	49-50	51-52	53-54	55-56	57-58	63-64		71-72
25-26																										1
27-28																										5
29-32																										7
33-36																										19
37-40																										8
41-44																										35
45-48																										37
49-52																										48
53-56																										48
57-60																										55
61-64																										77
65-68																										84
69-72																										97
73-76																										71
77-80																										66
81-84																										44
85-88																										13
89-92																										4
93-96																										1
101-104																										2
109-112																										1
113-116																										1
Totals	1	2	3	19	94	115	104	84	76	60	46	27	32	16	7	11	5	5	6	3	2	1	1	2	1	723

$\sigma_{\text{death}} = 3.4322,$

$\sigma_{\text{marriage}} = 3.5867$

$r = .2219 \pm .0236.$

 $\sigma_{\text{death}} = 3.4322, \quad \sigma_{\text{marriage}} = 3.5867 \quad r = .2219 \pm .0236.$

TABLE XIII.

Age at marriage of father and age at marriage of son.

	Father												Son												Totals
	13-14	15-16	17-18	19-20	21-22	23-24	25-26	27-28	29-30	31-32	33-34	35-36	37-38	39-40	41-42	43-44	45-46	47-48	49-50	51-52	53-54	55-56	57-58	63-64	73-74
13-14	1																								1
15-16		1																							3
17-18			1																						7
19-20				1																					28
21-22					1																				108
23-24						1																			102
25-26							1																		96
27-28								1																	75
29-30									1																42
31-32										1															33
33-34											1														29
35-36												1													18
37-38													1												9
39-40														1											5
41-42															1										2
43-44																1									6
45-46																	1								1
47-48																		1							1
49-50																			1						4
51-52																				1					1
53-54																					1				713
55-56																									1
57-58																									1
59-60																									4
Totals	1	2	3	18	89	112	105	84	74	59	47	28	31	16	7	11	5	5	6	3	2	2	1	2	1

 $\sigma_{\text{father}} = 3.4747, \quad \sigma_{\text{son}} = 3.4683, \quad r = -0.027 \pm .0243.$

TABLE XIV.
Age of father at marriage and age at death of eldest son.

Death of son	Marriage of father																Totals
	Under 20	21-24	25-29	30-34	35-39	40-44	45-49	50-54	55-59	60-64	65-69	70-74	75-79	80-84	85-89	90-94	
13-14																	4
15-16																	3
17-18																	6
19-20																	11
21-22																	6
23-24																	11
25-26																	6
27-28																	11
29-30																	6
31-32																	11
33-34																	6
35-36																	11
37-38																	6
39-40																	11
41-42																	6
43-44																	11
45-46																	6
47-48																	11
49-50																	6
53-54																	11
55-56																	6
57-58																	11
59-60																	6
Totals	1	3	8	30	115	103	99	92	75	47	44	34	32	18	13	10	746

$\sigma_{\text{marriage}} = 3.5429$, $\sigma_{\text{death}} = 3.7561$, $r = -.0298 \pm .0242$.

Only two of these coefficients require note as the other four must necessarily be large owing to the nature of the data. r_{12} is negative, though small; as all the others are positive, it will be larger and still negative when the other variables are made constant. r_{24} is small but positive, this is due not to a transmitted tendency, but to the fact that the data cover a large period of time during which the marriage conventions have altered, and hence a slight positive association is produced. The restriction of the data as already narrated was not sufficiently stringent to remove it. Making the age of the father at death and marriage constant, we have:

$${}_{34}r_{12} = - \cdot 1107 \pm \cdot 0211.$$

It would thus appear that a negative association exists between the age of the father when the son is born and the age at which the latter marries. It must be admitted that the value obtained is open to doubt; it is questionable as to whether it is really necessary to make the age of the father at death constant in all cases, for it may be, that only in the instances in which the son is a minor at the father's death is correction needful. Still, if we suppose that the true value lies between $- \cdot 1107$ and $- \cdot 0175$, the total and partial values, it would still suggest that a negative association exists.

A seventh table has been added to the above series, namely, Age of father at marriage and age at death of eldest son; in this case $r = - \cdot 0298 \pm \cdot 0246$, this lends support to the value of the partial r found for Age of father at birth of son and age of son at death, namely $r = - \cdot 0434 \pm \cdot 0239$.

The following series of observations are of some interest if the data can be said to be free from fallacy.

The information was obtained from such parents as attended the examination of their children under the provisions of the Education Act of 1907. As the particulars under consideration were limited to one age period, each pair was recorded once only. It is to be noted, however, that when information is obtained through the medium of offspring respecting characters pertaining to the parents, it is subject to certain fallacies that have already been mentioned and it may be that the following results are dependent on the number of surviving children possessed by certain of the categories into which the data were divided, rather than a consequence of the existence of a relationship between the age of the parents at birth of mates. This association is to be expected if age at birth influences age at marriage, for if a man

mates early because his father was old when he was born, that man must possess qualities which differentiate him from others, and in so far as assortative mating is an accepted fact a positive correlation should exist between the ages of parents at birth of mates.

The point is one of interest and it would be advisable that this portion of the enquiry should be re-examined through the material directly obtained by the Registrars on the occasion of the registration of a marriage. The constants for these observations are :

Age of mother at birth of husband, and age of mother at birth of wife :

$$r = .01798 \pm .0194.$$

It may be doubted whether it is justifiable to regard age as a simple variable or to consider definite periods of time as representative of changes that occur in an organism, dependent upon duration; that is to say, it is doubtful whether the period designated by the 50th year measures something similar to that of the 20th. If this is the case then a contingency table would give a better reflection of the bias if such exists, than the fitting of a straight line to a series of means.

The coefficient of contingency $C_2 = .1654$. If corrected

$$C_2 = .0964 \pm .0214$$

(approximate only, computed by $\frac{1 - C_2}{\sqrt{\eta}} .67449$).

If we evaluate the correlation ratio

$$\eta \text{ (husbands' mothers)} = .06496 \pm .0181.$$

The linear prediction formula is $Y = 28.484 + .01831 X$ years.

We conclude that a small association does exist but that the regression is probably not strictly linear. Turning now to the age of the father, we have the following coefficients :

Age of father at birth of husband, and age of father at birth of wife :

$$r = .0524 \pm .0221.$$

Coefficient of contingency $C_2 = .1954 \pm .02799$.

If corrected $C_2 = .0931$ and the correlation ratio for arrays of husbands' fathers

$$\eta = .1233.$$

If corrected

$$\eta = .0997 \pm .02184.$$

The association between age of father at birth of husband and age of father at birth of wife is more marked than in the case of mothers.

TABLE XV.

Age of father at birth of husband.

Age of father at birth of wife	Age of father at birth of husband.						Totals
	20 & under	21—25	26—30	31—35	36—40	41 & over	
20 & under	2	8	9	10	5	3	37
21—25	9	46	39	28	36	25	183
25—30	11	50	69	47	30	22	229
31—35	8	52	81	47	22	19	229
36—40	5	26	28	30	32	15	136*
41 & over	6	24	34	33	27	15	139
Totals ..	41	206	260	195	152	99	953

 $C_{2(r)} = .1954 \pm .02799$. Order of probability about .1. $\sigma_{\text{wife}} = 1.1411$. $\sigma_{\text{husb.}} = 1.3717$.

 $r = .0524$. $\eta_{\text{wife}} = .1223$. $\frac{\sqrt{n}}{.67449} \frac{1}{2} \sqrt{\eta^2 - r^2} = 2.5287$.

* Calculated by Blakeman's Formula.

TABLE XVI.

Age of mother at birth of husband.

Age of mother at birth of wife	Age of mother at birth of husband.						Totals
	20 & under	21—25	26—30	31—35	36—40	41 & over	
20 & under	23	32	28	23	20	8	134
21—25	41	101	95	62	35	23	357
26—30	49	91	97	51	39	16	343
31—35	26	90	80	58	25	13	292
36—40	22	31	46	39	19	6	163
41 & over	9	23	30	13	15	5	95
Totals ..	170	368	376	246	153	71	1384

 $C_r = .1654 \pm .0214$. $\sigma_{\text{husb.}} = 1.3554$. $\sigma_{\text{wife}} = 1.3801$. $r = .01798 \pm .0194$.

 $\eta_{\text{husb.}} = .06496$. Linearity = 1.7. Equation $Y = 28.484 + .01831 X$.

If then it can be assumed that these coefficients are not dependent on any statistical fallacy, they lend support to the original proposition, namely that the age at which mating occurs is to some extent determined by the ages of the parents when the parties concerned were born.

I am aware of, and have done my best to emphasise, the defects inherent in the data used in the present enquiry, and a necessary consequence of these imperfections is that several inferences can only be drawn with hesitation.

I think, however, that the concordance of the various results is sufficient to allow me to conclude that

(1) The ages of parents at the time of birth of their offspring are sensibly correlated with the latter's length of life, the sign of the

association being negative. The absolute value of the correlation is, however, small.

(2) This unfavourable influence acts principally at the beginning of life, becomes less marked during the adolescent period and perhaps again becomes prominent at the end of life.

I hope in a subsequent communication to deal with other aspects of this problem.

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THE STERILITY OF NORMAL URINE IN MAN.

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It appears to be widely believed that normal human urine as voided is sterile, and that the fact of sterility is capable of ready demonstration.

The main grounds for this belief are the well-known experiments on the subject by Lord Lister, and the innumerable negative cultural results that have since been obtained after inoculation of laboratory media with unincubated urine or its centrifugal deposits.

When, however, the experiments of Lord Lister are carefully studied it is at once apparent that they do not prove the sterility of normal urine, as he would have been to-day the first to acknowledge. For example many of the specimens of urine that he examined for evidence of sterility were allowed to stand at room temperature for considerable periods, and if no organisms were then found, or if the urine remained clear, it was assumed that none were present.

Evidence of this nature, however, is on theoretical grounds hardly admissible because incubation of the urine at body temperature might conceivably be necessary to allow of multiplication of infecting organisms to take place. This possible explanation of Lord Lister's deductions from his experiments on the sterility of normal urine was therefore put to the following simple test.

Experiment 1.

An ordinary specimen of male urine from a healthy subject was received into two sterile flasks, which were at once closed with sterile rubber corks. Flask 1 was incubated at body temperature for 48 hours, and its contents were then found to be turbid from the presence of large Gram positive dividing cocci. Flask 2 was allowed to stand at room temperature for 10 days, and at the end of this time the urine was found to be perfectly clear. This flask was then placed in the incubator for 48 hours, and the contained urine was at the end of this time found to be turbid from the presence of large Gram positive dividing cocci. Two peptone agar tubes, which had each been inoculated with the invisible centrifuged deposit of 5·0 c.c. of the clear

urine immediately after its collection, were free from colonies at the end of a period of 14 days, during which incubation at 37·0° C. had been allowed to proceed without interruption.

From this experiment it is clear that the absence of turbidity in unincubated urine after a lapse of several days does not necessarily indicate its sterility, and evidence of this nature therefore cannot be held to be satisfactory.

There remains for consideration the evidence afforded by the negative cultural results that often follow the inoculation of laboratory media with unincubated urine or its centrifuged deposits.

On theoretical grounds it would appear unsafe to assume the sterility of specimens of normal urine merely from failure to infect the ordinary laboratory media. For example, a given specimen might contain organisms which were unable to adapt themselves to a new environment, or could only do so if firmly established on urine first. And this would particularly apply to attempts to inoculate solid media. Moreover, the possibility of an infection of the total volume of urine discharged, for example, in 24 hours could hardly be excluded by failure to cultivate from the small fractional samples usually placed into tubes containing nutrient agar, or capable of reception by the ordinary centrifuge. And finally the degree of infection of normal urine might be so slight as to escape detection in the case of centrifuged deposits from clear urine unless these were transferred without loss to the medium employed as indicator. And this is a difficult feat to achieve.

Notwithstanding these theoretical objections to the use of laboratory media as indicators of the absence of infection of normal urine, the frequent negative results obtained are widely quoted as evidence of its absolute sterility. The value of laboratory media as indicators of sterility was therefore submitted to the following tests.

Experiment 2.

Clear urine from a healthy male was discharged into two sterile tubes, each receiving about 15·0 c.c. Tube 1 plugged with sterile wool was incubated at body temperature for 48 hours, and was then found to contain urine turbid from the presence of dividing cocci in groups. Tube 2 immediately after filling was centrifuged for 15 minutes in a high speed machine, and the supernatant urine carefully pipetted off. As much as possible of the slight deposit of mucus was then transferred in a platinum loop to two agar tubes, which were incubated for 14 days, and examined daily. One tube remained sterile throughout, the second showing one colony of cocci at the end of 76 hours, no further colonies appearing within 14 days.

From this experiment, confirming a similar observation in Experiment 1, it is clear that the inoculation of a solid medium such as nutrient agar with the centrifuged deposit from normal urine cannot be relied on to demonstrate its sterility.

Experiment 3.

The same conditions were observed as in Experiment 2 with the exception that the centrifuged deposit was transferred to two tubes of broth. The control tube of uncentrifuged urine and one of the tubes of broth were found to be turbid with growth after incubation for 48 hours, whilst the second tube of broth was still clear at the end of 21 days.

This experiment suggests that it is not safe to assume the sterility of a sample of normal urine from the absence of growth in broth inoculated with the centrifuged deposit of clear urine.

It may, however, be reasonably objected that the simplest method of determining if a given specimen of urine is sterile or not is to inoculate broth or agar, or other suitable medium, with fractional volumes of uncentrifuged urine. The validity of this objection was therefore tested.

Experiment 4.

Clear urine was discharged into a sterile flask, and 1.0 c.c. was at once pipetted over the surface of the medium in each of three agar tubes, the flask and the three tubes being subsequently incubated at body temperature for 76 hours, care being taken to prevent movement of the urine in the agar tubes during incubation. At the end of 76 hours the surfaces of agar were free from colonies, whilst the urine in the flask and in the three tubes was turbid with dividing cocci.

This experiment shows that if an inoculum of urine in an agar tube be undisturbed during incubation a fallacious result may be obtained if the laboratory medium alone be used as the indicator of sterility of the inoculum. And it also suggests that the urine is a more sensitive indicator of its own infection, as might be expected from its liquid nature, than is nutrient agar.

Experiment 5.

Clear urine from a healthy male was next discharged into a sterile test tube, and two tubes of agar were each inoculated with 1.0 c.c. of the urine obtained, a different pipette being used for each inoculum. During incubation the urine was repeatedly flushed over the surface of the agar, and in 48 hours each surface was covered with countless colonies of large dividing cocci. The control tube containing incubated urine alone also contained at the end of 48 hours large dividing cocci.

This experiment suggests that once the organisms found had firmly established themselves on urine they had no difficulty in adapting themselves to the solid medium if opportunity were allowed for re-inoculation.

Experiment 6.

Clear urine was then discharged direct into tubes of broth, and in 48 hours the incubated mixture was in the three tubes inoculated turbid with large dividing cocci.

Taken as a whole these six experiments suggest that the evidence hitherto relied on in favour of the sterility of normal urine should be abandoned, and that the fact of sterility of normal urine, if it be a fact, still awaits demonstration. And they also suggest that urine when incubated may be a better and more convenient indicator of its infection in the fresh state than the ordinary laboratory media whether liquid or solid.

If, however, incubated urine be used as the indicator of its absolute sterility, complete exclusion of extraneous organisms during the process of collection is essential. Unfortunately this is not an easy matter owing to our ignorance of the commonest source of extraneous infection, the difficulty of collecting human urine in such a way as to exclude it, and the time required in any given case to determine whether organisms found in, or cultivated from, the urine are extraneous or not. By extraneous infection is here meant infection which occurs after the escape of urine from the bladder. The possible sources of infection of male urine (with which alone this paper is concerned) occurring during and after discharge appear to be the urethra including the vestibule, the surface of the glans and of the lips of the meatus, the air through which the urine is collected, and the receiving flask. In all the experiments here cited, unless otherwise stated, infection from the receiving flask was excluded by exposing it to dry heat at 180.0°C ., each flask being plugged with wool before being heated. Infection from the urethra and vestibule and from the lips of the meatus was excluded as far as possible by adoption of the following technique. The surfaces of the glans and of the lips of the meatus were washed with sterile wool soaked in a 1 in 1000 solution of perchloride of mercury in water. The glans was then immersed in this solution for one minute, a few ounces of urine being finally discharged under the surface of the solution in order to flush the urethra and vestibule. Assuming these precautions to be efficient, the only two sources of extraneous infection which remain are the urethra, including the vestibule in so far as it cannot be

cleansed by the flushing referred to of organisms that are supposed normally to inhabit it, and the air through which discharge takes place.

In spite of these precautions however, as already shown, it was not found possible to obtain a sterile specimen of urine. The two methods of exclusion of extraneous infection usually relied on by different observers are the serial method of collection and the catheter method. Both these methods were, therefore, carefully tested in order to determine if by the adoption of either of them sterile specimens of normal urine could be obtained, using as the indicator of sterility the incubated urine itself.

By the serial method is here meant collection into a numbered series of flasks, or other receptacle, only the last members of the series being reserved for study.

Experiment 7.

After careful washing of the genital surfaces and after preliminary flushing of the urethra under perchloride of mercury, urine was discharged through air by a healthy male into a series of narrow mouthed flasks numbered 1 to 4. After incubation for 48 hours each flask was found to be turbid with organisms. Each flask contained about 100·0 c.c. of urine.

This experiment was repeated by the same individual five times in one laboratory, once in a second laboratory and twice in a private house. Of the 24 flasks incubated not one remained sterile at the end of three days. All were turbid, the organisms found in each series being constant to the site of collection of each series, but differing in each site. One series, for example, in one laboratory gave small cocci only in groups, a second in a different laboratory gave a short bacillus, cocci in groups and chains, a streptothrix and yeast-like cells, and a third series gave large cocci alone.

Experiment 8.

In this experiment urine was discharged by a healthy male into a series of four numbered filter flasks, the side piece being plugged with sterile wool. In each case the glans was placed within the mouth of the flask to exclude air as far as possible, and each flask was finally closed with a sterile rubber cork. At the end of a period of incubation lasting ten days each flask, containing about 60·0 c.c. of urine, was found to be heavily infected. This experiment was repeated 15 times by ten healthy subjects with varying volumes of urine, extreme care being taken with the preliminary toilet of the genital surfaces and vestibule. Of a total of 64 flasks incubated for 14 days not one contained sterile urine at the end of that time. Great irregularity was noted as to the time of appearance of turbidity, which was often independent of the position of a flask in the series, even when the volumes were constant.

Experiment 9.

Into three series of flasks, each series containing 12 members, were collected, in the same way as in Experiment 8, volumes of urine varying from 10 to 40 c.c. Of the 36 specimens of urine incubated not one was found to be sterile at the end of 14 days.

Thus out of a total of 128 flasks of normal urine in these experiments a sterile specimen was not once obtained.

There are three alternative interpretations of these results:

1. Normal urine, contrary to general belief, is not sterile when it leaves the bladder, that is, before it enters the urethra.

2. The serial method of collection cannot be trusted to exclude urethral organisms from urine during its passage along the urethra, assuming such organisms to exist in the healthy subject.

3. A common source of extraneous infection of urine is the air through which it passes after it has left the urethra.

The experiments, in other words, throw no certain light on the source or time of infection of the various samples of urine examined, though from Experiment 7 the possibility of air infection is certainly suggested. They do, however, clearly show that the serial method of collection through unsterilized air is of no value in attempting to obtain sterile specimens of normal urine.

The catheter method of collection was next examined.

Experiment 10.

Specimens of normal urine from ten healthy males were collected by catheterization, the urine being discharged through air in the ordinary way, the free end of the catheter being finally placed inside sterile flasks. The catheters were sterilized before use in liquid paraffin in the autoclave at a temperature of 125·0° C. in order to avoid the necessity for lubrication after sterilization¹. In each case the first few ounces of urine discharged were rejected before the flasks were filled. After incubation of ten specimens obtained in this way turbidity due to the presence of organisms was present in all at the end of 14 days.

This experiment shows that the catheter method of collection as generally employed is no more reliable than is the serial method in securing sterile specimens of normal urine.

An attempt was therefore made to obtain a sterile catheter specimen by only allowing the urine to pass through sterilized air as it leaves the catheter.

Experiment 11.

A catheter specimen of urine was collected from a healthy male, the free end of the catheter, fitted with a clamp, being attached to the side piece of a filter flask before sterilization, the mouth being closed with a sterile wool plug. As soon as the

¹ Absolute sterilization being impossible to ensure by this method in the case of the presence of spore-bearing organisms, these were carefully searched for in the catheter specimens of urine obtained. Only non spore-bearing organisms were however cultivated.

specimen was obtained, but before withdrawal of the catheter from the bladder, the clamp was closed and the catheter cut off. After incubation for 21 days the urine was found to be clear, and appeared to be sterile so far as could be judged from film preparations of the centrifuged deposit and from inoculation of broth.

Immediately after separation of the catheter from the flask a few ounces of urine were drawn through unsterilized air into a second flask, and in 48 hours the urine was found to be turbid with growth of large dividing cocci.

This experiment, taken alone, is of little value as evidence of the sterility of normal urine in general, but it suggests that a normal specimen can be obtained in an apparently sterile condition if passed through sterile air.

That unsterilized air may be a factor in extraneous infection of urine during its collection is also suggested by an experiment of a different nature.

Experiment 12.

A specimen of urine passed in the ordinary way without any preliminary toilet was collected in a sterile flask. Twenty c.c. of the urine were then transferred in a sterile pipette into a test tube. This was at once boiled, and when cool ten c.c. were poured through air into a second tube. The flask and the two tubes were then placed in the incubator for 48 hours. The flask and the tube containing the poured urine were both infected with large Gram positive dividing cocci, whilst the second tube containing the boiled urine which had not been poured remained clear and sterile at the end of a month. This experiment was repeated three times with the same results.

CONCLUSIONS.

1. As an index of the sterility of normal urine absence of growth on the ordinary laboratory media inoculated with fresh urine or its centrifuged deposit is of little or no value, except in the case of broth inoculated with uncentrifuged urine.

2. The use of incubated urine as the medium of growth shows it to be a highly sensitive and reliable indicator of infection with organisms that appear to be extraneous in the sense defined.

3. It is difficult, if not impossible, to obtain sterile specimens of normal urine by the serial method or the catheter method of collection as ordinarily carried out.

4. Unsterilized air through which urine passes during the process of collection appears to be a frequent source of the extraneous infection of urine, whether the catheter or serial methods be employed or not.

5. The sterility of normal urine in man still awaits demonstration.

SUPPLEMENTARY NOTE BY EDWARD C. HORT
AND W. W. INGRAM.

Working in conjunction we have repeated on a large scale involving several hundred observations the experiments above described, and have without exception obtained the same essential results.

It appears that the most convenient method of determining whether normal human urine is or is not sterile before it leaves the bladder would be to obtain a sample by suprapubic puncture during life. This procedure has obvious disadvantages in man. We have therefore applied a modification of the method to animals by puncturing the bladder immediately after death with the following results:

Owing to the kindness of the authorities of the Metropolitan Cattle Market we were able to examine the bladder urine of fourteen animals. Immediately after the death of the animals the bladder was exposed, its surface being then seared with a hot iron. A sharp sterile glass pipette was then pushed through the wall of the bladder and a few cubic centimetres of urine drawn up. The pipette was in each case at once sealed in the flame and within an hour placed in the incubator and kept there for two and a half months at a temperature of 37.0°C. Companion pipettes were withdrawn from the incubator at intervals and examined. The contents of these pipettes and of those kept in the incubator for two and a half months were perfectly clear and in no case could organisms be discovered in the centrifuged deposits, and in no case did inoculation of laboratory media with volumes of the incubated urine reveal infection.

We then repeated these observations with the urine of twelve apparently healthy guinea pigs, using the same technique. In each case the specimens examined remained sterile as far as could be judged by examination of the centrifuged deposit and by attempts at cultivation on the ordinary laboratory media inoculated with the incubated urine.

We conclude from these experiments that the normal urine of cattle, sheep and guinea pigs, provided that they are healthy, is probably sterile, and that normal human urine is also probably sterile. We have, however, not yet been able to prove the sterility of normal human urine.

EXPERIMENTAL PURPURA

(SECOND COMMUNICATION)

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EXPERIMENTAL PURPURA.

IN a preliminary communication by one of us (Ledingham¹) to this journal on June 13th, 1914, the production of purpura hæmorrhagica in guinea-pigs, after inoculation with an antiserum prepared by immunising rabbits with guinea-pig blood plates, was placed on record. The present paper deals mainly with conjoint work carried out in June and July of last year, together with additional experimental data since obtained by one of us (L.) in the absence of the other (B.) on military service. The blood changes in guinea-pigs inoculated with anti-plate serum have been followed out in detail, and we propose in the first place to submit data on this point. A description will follow of experimental purpura in the rabbit and rat induced by inoculation with their respective anti-plate sera.

Blood Changes in Guinea-pigs after Inoculation with Anti-guinea-pig-plate Serum.

Notes on technique.—The blood plates were counted in a Thoma-Zeiss chamber, the blood from the ear being received into citrate solution containing brilliant cresyl blue which showed up the blood plates very clearly. The figures for the plate content per c.mm. obtained by this method in a series of normal guinea-pigs were as follows: 856,000, 752,000, 340,000, 640,000, 672,000, 688,000, 590,000, 696,000, 664,000, 592,000 (the latter four obtained from the same animal on four successive days), 592,000, and 584,000.

¹ Ledingham (19 4): The Experimental Production of Purpura in Animals, &c., *THE LANCET*, vol. i., 1914, p. 1673.

The average of nine animals works out about 638,000 per c.mm. The hæmoglobin was estimated by Haldane's instrument. Attempts were also made to obtain coagulation times, but as we have not satisfied ourselves that the methods employed yield reliable results, at least so far as the guinea-pig is concerned, we refrain at present from alluding to them. The statement in the preliminary communication that the clotting is delayed rested entirely on observations of the bleeding time after puncture. Prolongation of the bleeding-time after puncture of the ear vein is a very marked feature in experimental guinea-pig purpura (as Duke asserts to be the case in human purpura), but this function may prove to have no direct relation to the coagulation time as ordinarily understood and calculated.

The three animals, B 3, B 4, and B 5, whose blood changes, &c., have been followed daily, received each one dose of anti-plate serum (1.5 c.c.) subcutaneously immediately after the control normal samples had been taken. The animals were purposely chosen of large size (over 400 gm.), so that a lengthy observation period would be available before the fatal conclusion (or possibly recovery) resulted. It had previously been determined that in animals of 250 gm. a dose of 1 c.c. of a particular batch of anti-plate serum had produced death in three, four, or five days with typical purpuric lesions. The data obtained from these three large animals, together with notes of the general condition, are found in Table I.

Remarks on Table I.—It will be observed from the table that two of the animals died in 4 and 8 days respectively, while the third animal (B 3), whose initial weight was 450 gm., made a complete recovery after exhibition of purpuric skin lesions. On July 14th—i.e., 36 days after the first dose—it received a second dose of 2 c.c. of the serum. The reaction to this second dose was very slight, the blood plates only, showing a marked fall, while the purpuric skin spots were few. It is impossible here to analyse the blood changes in detail, but certain points deserve notice.

1. The total leucocyte count is not appreciably



affected, though there is a distinct tendency to relative lymphocytosis (see the column of differential counts). 2. The blood plates are the elements which suffer the earliest and promptest reduction. Except in the case of B5, which showed an abnormally low initial plate content, the plates fell to one-sixth to one-tenth of their initial figure. In animal B3, which recovered, the rapid regeneration of red cells and hæmoglobin is well seen. In the last two columns data are appended with reference to the presence of hæm-agglutination in the shed blood, the minute changes in the red cells, and the general condition of the animals.

Post-mortem appearances of the above three animals. B 3.—Killed for examination on July 18th. A few skin hæmorrhages present at site of last inoculation. A few groups of faded punctiform hæmorrhages in stomach wall. No petechiæ on heart or lungs. Lymph glands not of hæmolymph character. Urine not stained with hæmoglobin.

B 4.—Dead June 16th. This animal had shown numerous skin hæmorrhages during the early days following inoculation of the serum. At necropsy these had faded and skin hæmorrhages were found only in the neighbourhood of the inoculation site. The red colour of the lymph glands was disappearing. Several hæmorrhages in the lungs and very numerous petechiæ in intestine. Stomach mucosa showed two round patches—one about the size of a sixpence, the other half that size—studded with small punctiform hæmorrhages. There was no ulceration. Liver yellowish. Spleen large, dark red, and friable. Kidneys and adrenals apparently normal.

B 5.—Dead June 12th. Numerous hæmorrhages at site of inoculation and a few scattered about elsewhere. Only a few lymph glands show the hæmolymph change. No hæmorrhages in intestine. Liver pale and yellowish. Spleen greatly enlarged and friable. Urine blood-stained. A few small hæmorrhages in testicles. *Both eyes show cataract.*

Blood Changes in Smaller Animals.

To complete this part of the subject tables are also appended showing the variations in the red cells, blood plates, hæmoglobin, &c., in three guinea-pigs of less weight. Each was inoculated on June 30th,

1914, with 1 c.c. of anti-plate serum subcutaneously.
(See Tables II. A, II. B, and II. C).

TABLE II.A.—*Guinea-pig 8.*

Date.	Red cells.	Plates.	Hb.	Weight.
June 30th*	5,744,000	640,000	96%	240 gm.
July 1st	5,944,000	512,000	94%	220 "
" 2nd	5,130,000	320,000	80%	210 "
" 3rd	664,000	98,000	—	200 "

Death between 1 and 2 P.M. on July 3rd.

* Received 1 c.c. anti-plate serum subcutaneously.

Notes on blood picture and general condition.—July 1st: Blood picture normal except for one or two red cells showing polychromatophilia. Well-marked hæmagglutination. Pig lively. Distinct infiltrate over abdomen. July 2nd: Some irregularity in shape of red cells. Megalocytes of medium size, fairly numerous, mostly showing polychromatophilia. Some red cell shadows. Pig seems ill. Infiltrate softening. Some skin hæmorrhages. Gums of fairly good colour. July 3rd: Hæmagglutination of shed blood most pronounced. Great irregularity in size of red cells. Megalocytes showing polychromatophilia, very numerous; poikilocytes; no punctate basophilia. A few normoblasts, some showing pyknosis and karyorrhexis of nucleus. A few red cells with "Jolly" bodies. Pig cold and collapsed; blood thin and watery; no eye changes observed. Post mortem: Gelatinous blood-stained infiltrate over abdomen and thorax. A fair number of small punctiform hæmorrhages in skin. Numerous hæmorrhages in subcutaneous tissue. No eye changes. Spleen dark red and friable. Bladder filled with dark, blood-stained urine. Testicles showed several large hæmorrhages. No hæmorrhages in stomach or intestines. Lymph glands of red hæmolymp character. Bone marrow dark red.

TABLE II.B.—*Guinea-pig 9.*

Date.	Red cells.	Plates.	Hb.	Weight.
June 30th*	6,620,000	672,000	93%	260 gm.
July 1st	5,952,000	480,000	96%	260 "
" 2nd	5,616,000	272,000	84%	240 "
" 3rd	720,000	392,000	16%	230 "

Death between 1 and 2 P.M. on July 3rd.

* At 5 P.M. received 1 c.c. anti-plate serum subcutaneously.

Notes on blood picture and general condition.—July 1st: Pig lively. Well-marked firm infiltrate over abdomen and extending forward to thorax. Blood picture normal except for one or two red cells showing polychromatophilia. July 2nd: Hæmagglutination well marked. Some red-cell shadows. Irregularity in size of red cells commencing. Megalocytes of medium size showing polychromatophilia, but not numerous. Animal ill. Infiltrate softening; some skin hæmorrhages; gums of good colour. July 3rd: Hæmagglutination extreme. Blood very watery; oozing after vein puncture. Irregularity in size of red cells. Megalocytes showing polychromatophilia; a few deeply basophile red cells; some poikilocytes. A few normoblasts, some showing punctate basophilia of cytoplasm. Pig collapsed and cold. No eye changes visible. Post mortem: A few small punctiform hæmorrhages in skin. Glands do not show hemolymph change. No eye changes. Bladder contained dark blood-stained urine. No hæmorrhages in internal organs.

TABLE II.C.—Guinea-pig 10.

Date.	Red cells.	Plates.	Hb.	Weight.
June 30th*	6,680,000	688,000	102%	360 gm.
July 1st	6,170,000	384,000	100%	320 "
" 2nd	5,504,000	328,000	84%	300 "

Death during night of July 2nd-3rd.

* At 5 P.M. received 1 c.c. anti-plate serum subcutaneously.

Notes on blood picture and general condition.—July 1st: Blood picture normal. Hæmagglutination well marked. Pig lively. Infiltrate not very well marked. July 2nd: Irregularity in size of red cells. Fair number of megalocytes, mostly showing polychromatophilia. Some red-cell shadows. Pig not well. Infiltrate more marked, but softer. Some skin hæmorrhages, one about the size of a threepenny-piece. Post mortem: Well-marked gelatinous blood-stained infiltrate. Numerous small punctiform hæmorrhages in skin. Glands of hemolymph character. A few small hæmorrhages in lungs and in wall of left ventricle. Spleen much enlarged and friable. One or two minute petechiæ in pancreas. Bladder full of blood-stained urine. Numerous hæmorrhages in wall of large gut and in testicles.

Remarks on Tables II.A, II.B, and II.C.—These three animals, whose initial weights were 240 gm., 260 gm., and 360 gm., did not survive beyond the third day. All showed pronounced purpura. The

effect of the serum on the blood elements is essentially similar to that obtained in the case of the larger animals of Table I. Notes regarding the minute changes in the red cells, the general condition of the animals during life, and the post-mortem appearances are appended to the tables.

Control Experiments with other Sera: Action of Anti-leucocytic Serum.

This serum was prepared by immunising rabbits with leucocytes obtained from the peritoneal exudates of guinea-pigs which were killed 4 hours after an intraperitoneal inoculation of 5-10 c.c. of sterile broth. Such exudates, as is well known, consist almost entirely of polymorphonuclear leucocytes, and if great care is taken in manipulation of the animals after death the risk of admixture with red cells can be very largely avoided. The exudates are taken up in citrated saline and thereafter washed in normal saline. The serum of the rabbits so immunised proved to be quite as fatal to guinea-pigs as the anti-plate serum, but did not produce purpuric phenomena. We append two tables (Nos. III.A and III.B) showing the action of the serum on the blood elements throughout the course of the experimentally induced disease.

TABLE III.A.—*Guinea-pig 15.*

Date.	Red cells.	Plates.	Leucocytes.	Differential count.†				Hb.	Weight.
				P.	E.	L.	Ma		
July 8th*	6,128,000	592,000	10,200	40.5%	0.5%	58.5%	0.5%	100%	Gm. 290
„ 9th	4,440,000	456,000	2,900	3.0%	0.5%	96.0%	0.5%	80%	250
„ 10th	2,080,000	640,000	10,800	10.0%	1.0%	88.0%	1.0%	40%	230
„ 11th	?	?	?	25.0%	2.0%	72.5%	1.5%	?	220

Death at 3 P.M. on July 11th.

* At 5.30 P.M. received 1 c.c. anti-leucocyte serum subcutaneously.

† P = Polymorphonuclears. E = Eosinophiles. L = Lymphocytes.
Ma = Mast cells.

Blood picture and general condition.—July 9th: Red cells irregular in size; numerous polychromatophilic megalocytes; a few red cells showing "Jolly" bodies; no nucleated red cells. The polymorphonuclear leucocytes are degenerated, their protoplasm being vacuolated and the granules appearing as indistinct basophile knots in cytoplasm. Pig ill. Small infiltrate under skin; no skin hæmorrhages. July 10th: Large numbers of megalocytes; some microcytes; well-marked polychromatophilia; nucleated red cells (normoblasts) 1·5 per cent. of white cells. Some phagocytosis of red cells by mononuclears. General condition unchanged. No skin hæmorrhages. July 11th: Some red cells show punctate basophilia; nucleated red cells (normoblasts and megaloblasts) 21 per cent. of white cells. Neutrophile myelocytes present. Pig ill and collapsed; hæmoglobinuria; no eye changes. Post mortem: No skin hæmorrhages. Lymph glands appear normal. No hæmorrhages in internal organs. Spleen greatly enlarged and friable. Bladder full of blood-stained urine.

TABLE III.B.—Guinea-pig 16.

Date.	Red cells.	Plates.	Leucocytes.	P.	L.	Ma.	Hb.	Weight.
July 8th*	6,368,000	584,000	14,400	51·0%	49·0%	—	98·0%	Gm. 270
" 9th	5,560,000	643,000	2,600	3·0%	97·0%	—	90·0%	240
" 10th	2,630,000	640,000	8,000	6·6%	88·6%	3·3%	52·0%	220
" 11th	1,608,000	776,000	13,200	34·0%	67·5%	0·5%	22·0%	200

Pig dead at 6 P.M. on July 12th.

* At 5.30 P.M. received 1 c.c. anti-leucocyte serum subcutaneously.

Blood picture and general condition.—July 9th: Slight hæmagglutination. Irregularity in size of red cells; poikilocytes; polychromatophilic megalocytes; one nucleated red cell seen. Degeneration of polymorphonuclears as in No. 15. Pig seems ill. Very slight infiltrate; no skin hæmorrhages. July 10th: Marked irregularity in size of red cells; no nucleated red cells. Infiltrate soft; no skin hæmorrhages. July 11th: Many megalocytes showing polychromatophilia; some red cells with punctate basophilia; nucleated red cells (normoblasts) 3·5 per cent. of white cells. Phagocytosis of red cells observed. Myelocytes 4 per cent. (total neutrophiles, 34 per cent.). Pig very ill. No eye changes. Post mortem:

No skin hæmorrhages. No eye changes. No hæmorrhages in internal organs. Spleen slightly enlarged. Hæmoglobinuria. Liver yellowish.

It will be seen from the tables that while the red cells were very considerably affected, the blood plates were not appreciably altered in number. The most striking phenomenon in connexion with this serum is the great but temporary fall in the absolute number of leucocytes. In this fall, as the differential counts show, the polymorphonuclear cells are chiefly involved. No hæmorrhages were seen on the skin during life or in the organs after death. In both cases hæmoglobinuria was a well-marked feature.

Action of Anti-red-cell Serum (Ordinary Lytic Serum).

The effects of injecting lytic serum into the animal whose red cells have been used for immunisation have been studied by several workers, and notably by Muir and McNee (1912),² who have experimented with anti-rabbit red-cell serum prepared by immunisation of the goat with rabbit red cells. No record has been found of the production of purpuric lesions by such sera. It was, however, considered advisable to prepare a lytic serum for comparative tests, and for this purpose rabbits were immunised with washed guinea-pig red cells in the usual way. This serum has been tested on guinea-pigs only in a few cases so far. It is very lethal for guinea-pigs, but no evidence has been obtained that it can give rise to generalised purpura. As we shall see later, an anti-rat-red-cell serum proved to be equally devoid of purpura-producing effects when injected into rats, whereas the anti-rat-plate serum consistently produced purpura.

² Muir, R., and McNee: The Anæmia Produced by a Hæmolytic Serum, *Journal of Pathology and Bacteriology*, vol. xvi., p. 410.

The Question of Dosage of Anti-plate Serum.

To test this point four guinea-pigs, each exactly 250 gm. weight, received the following quantities of anti-plate serum subcutaneously in the dorsal region on June 2nd, 1914: 2 c.c., 1 c.c., 0.5 c.c., and 0.1 c.c. (volume in all cases made up to 2 c.c. with saline). The animal which received 2 c.c. died on June 6th, showing an enormous number of hæmorrhages, with petechiæ on the heart and lungs and double cataract. The animal which received 1 c.c. died on June 4th (i.e., after 42 hours). There was beautiful miliary purpura over the whole skin, with petechiæ on the heart and lungs. Cataract had not developed. The animal which received 0.5 c.c. developed, like the others, a local swelling which had practically disappeared on June 6th. It died ten days later, but showed no purpuric lesions either internally or externally. Very probably any such lesions that may have occurred and escaped detection in the early days after inoculation had faded. The animal which received 0.1 c.c. showed only very slight local swelling for a few days after inoculation, and remained well.

Experimental Purpura in the Rabbit.

An anti-rabbit-plate serum was prepared by immunising the goat with rabbit blood plates recovered from rabbit blood by a method similar to that employed in the case of guinea-pig blood. Each dose given to the goat consisted of the blood plates recovered from the mixed blood of 12 rabbits. About nine such injections were given. The serum of the goat was tested at various times during the course of immunisation, and it will be convenient to detail briefly the results obtained with this serum on various rabbits, as in certain cases highly interesting pathological lesions were found at necropsy. In the earlier cases of the series purpuric lesions in the skin were unfortunately not searched for.

Rabbit 1. Received on June 20th 4 c.c. anti-plate serum subcutaneously (from test bleeding of goat after three doses). June 21st: Considerable soft local swelling. June 23rd: Swelling very slight. Rabbit remained well till July 15th, when sudden death took place. Post mortem: Animal well nourished. Peritoneal cavity filled with clotted blood and extravasated faeces. *Two large fairly clean-cut perforations of the bowel just below caecum. A few purpuric spots in small intestine. Some faded purpuric spot in skin, especially that of hind limbs. Large hæmorrhages in the psoas muscle and in the muscles over tibia and fibula. Effusions of blood in the periosteal sheaths of these bones. Stomach mucosa showed a cluster of minute hæmorrhages, but there was no general purpura of the organ. Liver and lungs healthy.*

Rabbit 2.—Received on June 20th 1.5 c.c. intravenously (test bleeding after four doses). Remained well.

Rabbit 3.—Received on June 30th 2 c.c. intravenously (test bleeding after four doses). Remained well till July 3rd, when it was given a further dose of 3 c.c. intravenously. Death occurred two hours thereafter. Post mortem: *Perforated ulcer of colon adherent by film of lymph to abdominal wall. Purpuric patches round the ulcer. The colon contained another large ragged ulcer with greyish scurf on it. It was not perforated. In the stomach were two similar large non-perforated ulcers. Right lung collapsed and congested. Some free blood in right pleural cavity. The liver contained a few nodules of pseudotuberculosis, so that some doubt remains whether the late purpuric lesions met with may have been of bacterial origin. This is, however, unlikely.*

Rabbit 4.—Small Russian Received on July 6th 5 c.c. intraperitoneally (test bleeding after four doses). July 7th: Three or four purpuric spots in skin over sternum. July 8th: Very large number of purpuric spots on skin of abdomen and back, and especially of extremities. Petechiæ on ear. On the same day it received a further dose of 3 c.c. intraperitoneally. July 9th: Rabbit looks well. Received a third dose of 3 c.c. intravenously and died in convulsions five minutes afterwards. Post mortem: Enormous number of skin hæmorrhages. Miliary purpura of stomach. One or two petechiæ on epicardium, and a good number on large bowel and under kidney capsule. Very large number in skin covering fore and hind extremities and tail. No cataract. Large amount of free blood in peritoneal cavity. One or two reddish lymph glands in axilla and groin. Urine quite clear.

Rabbit 5.—Very small Russian weighing 440 gm. Received on August 27th 4 c.c. intraperitoneally (test bleeding after seven doses). August 28th: Animal looks fairly well. Numerous purpuric spots on skin of abdomen and extremities. Weight 431 gm. August 29th: Animal dead. Post mortem: Beautiful purpura of skin. *Extensive purpura of caecum and large bowel.* A few spots in small intestine and in stomach. Numerous petechiae in diaphragm.

Blood Changes in Experimental Purpura of the Rabbit.

We append three tables (Nos. IV. A, IV. B, and IV. C) in which are recorded the blood changes, &c., in the case of three rabbits inoculated intraperitoneally with the same dose (3 c.c.) of anti-rabbit-plate serum.

TABLE IV. A.—*Rabbit 33.*

Date.	Red cells.	Plates.	Leucocytes.	P.	E.	L.	Hb.	Coag. time.
July 15th*	5,792,000	648,000	12,200	28.0 %	2.0 %	70.0 %	80.0 %	1' 47"
„ 16th	4,864,000	100,000	10,400	54.5 %	0.5 %	45.0 %	66.0 %	2' 55"
„ 17th†	4,872,000	496,000	9,800	27.5 %	1.0 %	71.5 %	64.0 %	1' 45"

* At 4 P.M. received 3 c.c. anti-rabbit-plate serum intraperitoneally.

† Killed with ether to obtain specimen of skin.

Blood picture and general condition.—July 16th: Hæmagglutination present. Some irregularity in size of red cells; a few red cells showing polychromatophilia; one or two nucleated red cells seen. Rabbit quite lively. Bleeds very readily when ear vein is punctured; marked purpura over entire skin. July 17th: No nucleated red cells seen; a few showing polychromatophilia. Rabbit quite lively. No fresh outburst of purpura so far as can be detected. Post mortem: Extreme purpura of skin. Lymph glands not of frankly hæmolymp character. A few punctiform hæmorrhages in stomach and large gut. Some extensive hæmorrhages in muscles of thigh. No hæmoglobinuria.

TABLE IV.B.—*Rabbit 34, weight 920 gm.*

Date.	Red cells.	Plates.	Leuco-cytes.	P.	E.	L.	Ma.	Hb.	Coag. time.
July 20th*	5,424,000	672,000	12,400	15.0 %	0.5 %	83.5 %	1.0 %	80 %	0' 58"
„ 21st	5,016,000	106,000	14,400	22.0 %	—	77.5 %	0.5 %	70 %	2' 0"
„ 22nd	3,824,000	232,000	12,100	30.5 %	—	68.5 %	1.0 %	50 %	1' 5"
„ 23rd	3,960,000	454,000	7,800	25.0 %	0.5 %	74.5 %	—	56 %	—
„ 24th	4,344,000	752,000	8,400	—	—	—	—	58 %	—
„ 25th	4,184,000	760,000	18,000	—	—	—	—	58 %	—
„ 29th	4,416,000	800,000	10,000	—	—	—	—	70 %	—

* At 4.15 p.m. received 3 c.c. anti-rabbit-plate serum intraperitoneally.

Blood picture and general condition.—July 20th: Before injection one or two nucleated red cells were seen in film. July 21st: Little change in red cells; one normoblast seen. Animal drowsy and unwell. Hæmorrhages in skin, and specially marked in skin of ears. The flicking or rubbing of the ears produces an immediate crop of small punctiform hæmorrhages. July 22nd: Many red cells showing polychromatophilia; marked irregularity in size of red cells; no nucleated red cells seen. Rabbit quite lively. Hæmorrhages still present in skin. July 23rd: Marked irregularity in size of red cells; some large megalocytes showing polychromatophilia; no nucleated red cells seen. Animal's condition good. July 29th: Animal's condition good. Purpuric spots in skin mostly faded. A few still present on ears.

TABLE IV.C.—*Rabbit 35, weight 700 gm.*

Date.	Red cells.	Plates.	Leuco-cytes.	P.	E.	L.	Ma.	Hb.	Coag. time.
July 20th*	4,240,000	640,000	9,600	25.5 %	0.5 %	72.5 %	1.5 %	62 %	1' 2"
„ 21st	3,696,000	98,000	16,400	52.5 %	—	47.0 %	0.5 %	50 %	30"
„ 22nd	3,840,000	356,000	9,000	22.0 %	2.0 %	76.0 %	—	44 %	—
„ 23rd	3,856,000	472,000	6,600	50.0 %	0.5 %	49.5 %	—	48 %	—

Killed with ether at 2 p.m. on July 23rd.

* At 4.45 p.m. received 3 c.c. anti-rabbit-plate serum intraperitoneally.

Blood picture and general condition.—July 20th: Before injection no nucleated red cells were seen. July 21st: Hæmagglutination present but not marked. Some irregularity in size of red cells; some poikilocytes; no nucleated red cells. Animal ill. Hæmorrhages in skin, and especially in skin of ears. July 22nd: No nucleated red cells. Animal's condition unchanged. July 23rd: No nucleated red cells. Condition unchanged. Post mortem: Skin shows numerous hæmorrhages. Lymph glands appear normal. Numerous intramuscular hæmorrhages in diaphragm. No hæmorrhages in stomach or intestine; no hæmoglobinuria. Some hæmorrhages of moderate size in thigh muscles.

It will be seen that all three animals developed marked generalised purpura from which they recovered. They were, however, killed for examination purposes at various dates, and the post-mortem appearances are appended to the tables. The marked and early fall in the plate content corresponding with the early onset of purpuric skin phenomena will be noted. In these rabbits coagulation times were taken by the Dale-Laidlaw method,³ the blood being drawn into short capillary tubes, each containing a small shot. The data so obtained are included, but we are not satisfied that they can be depended upon in view of the variations we have obtained by this method in normal rabbits examined at short intervals. This question is reserved for further investigation.

Experimental Purpura in the Rat.

Rabbits were immunised with rat platelets recovered from fairly large quantities of rat blood. Each immunising dose consisted of the plates obtained from the mixed blood of 24 sewer rats. Three rabbits were immunised, two of which died during the course of immunisation. From the remaining rabbit a small test bleeding was taken a few days after it received its second dose. The serum so obtained was tested on a rat and found to be efficacious in producing purpura (see below). The rabbit received a third and a fourth dose

³ Dale, H. H., and Laidlaw, P. P.: A Simple Coagulometer, *Journal of Pathology and Bacteriology*, vol. xvi., p. 351.

of rat plates, but unfortunately a few minutes after the intravenous injection of the latter dose it developed marked anaphylactic symptoms. It was felt that the serum of such an animal would probably be largely deprived of its anti-plate elements owing to absorption by the large dose of fresh plates injected. The animal was, however, bled when moribund and the serum so obtained filtered. As we shall see, this serum possessed marked purpura-producing property.

Effect of Injecting Anti-rat-plate Serum into Rats.

Rat 1.—Received on Sept. 29th 2·5 c.c. subcutaneously in dorsal region near root of tail (from test bleeding after second dose). Sept. 30th: Numerous minute skin hæmorrhages round the area of inoculation and along the tail. None elsewhere. Oct. 1st: The animal now moribund was killed with ether. Post mortem: A large oval skin hæmorrhage was found at site of inoculation, with smaller petechiæ at the periphery. Numerous hæmorrhages in skin of tail. *A few in pyloric portion of stomach.* No generalised skin purpura. The result, however, showed that the serum from this early test bleeding possessed the purpura-producing property.

Rat 2.—Received on Oct. 13th, 1914, 3 c.c. subcutaneously (from moribund rabbit after its fourth dose). Oct. 14th: A few petechiæ noted on skin of hind limbs. Oct. 15th: Animal not examined. Oct. 16th: Animal very ill and drowsy; was accordingly killed with ether. Post mortem: Beautiful generalised skin purpura. Large hæmorrhage in muscles of one fore limb; marked purpura of tail. *Several small hæmorrhages in pyloric portion of stomach.* Lymph glands bright red in colour. No hæmoglobinuria. No cataract.

Rat 3.—Received on Oct. 14th 4 c.c. subcutaneously. Oct. 15th: Cluster of small hæmorrhages near site of inoculation. Oct. 16th: More hæmorrhages in the neighbourhood. Animal very ill and drowsy. Oct. 17th: Rat dead. Post mortem: Generalised skin purpura. Red lymph glands throughout. *Petechiæ in pyloric portion of stomach and throughout a considerable part of the small intestine, also in sigmoid and rectum.* Urine in bladder blood-stained.

On the whole, the lesions in rat purpura are essentially similar to those met with in experi-

mental guinea-pig purpura except that cataract, so frequently found in guinea-pigs dying of the experimental disease, has not so far been noted in rats. As mentioned above, the effect of anti-rat red-cell serum was investigated on rats. No purpuric lesions resulted.

Action of Heterologous Anti-plate Sera.

The question of the specificity of these anti-plate sera is of importance, and at various times control experiments in settlement of this point have been made. Invariably the result has been that the animal does not respond to the heterologous anti-plate serum—e.g., only slight inconvenience and certainly no purpura follow the injection of the guinea-pig with anti-rabbit-plate serum, and *vice versa*. Recently, being in possession of a supply of anti-guinea-pig-plate, anti-rabbit-plate, and anti-rat-plate sera, one of us has carried out the following experiment, which shows clearly that a heterologous anti-plate serum has no purpura-producing property.

TABLE V.

Rat A,	150 gm.,	received 7 12 1914,	3 c.c. anti-rat-plate serum.
„ B,	90 „	„ „	3 „ anti-guinea-pig-plate serum.
„ C,	140 „	„ „	4 „ anti-rabbit-plate serum.

(All inoculated subcutaneously.)

Guinea-pig A,	230 gm.,	received 7/12/1914,	3 c.c. anti-guinea-pig-plate serum.
„ B,	230 „	„ „	3 „ anti-rat-plate serum.
„ C,	230 „	„ „	4 „ anti-rabbit-plate serum.

(All inoculated subcutaneously.)

Rabbit A,	400 gm.,	received 7/12 1914,	4 c.c. anti-rabbit-plate serum.
„ B,	380 „	„ „	4 „ anti-guinea-pig-plate serum.
„ C,	380 „	„ „	4 „ anti-rat-plate serum.

All inoculated intraperitoneally.)

On Dec. 8th Rat A showed several purpuric spots near tail and also on legs. Other two rats showed nothing and appeared

quite lively. Guinea-pig A had an extensive hæmorrhagic infiltrate over the whole of the abdomen and did not appear well. The other two animals showed no purpura and were quite lively. Rabbit A had numerous purpuric spots all over its skin and was obviously ill. The other rabbits were quite lively and showed no purpura. On Dec. 9th the animals inoculated with the homologous sera were still more drowsy and listless. Other animals appeared quite well. On Dec. 10th Guinea-pig A and Rabbit A dead.

Post mortem: Guinea-pig A showed beautiful skin purpura, extensive œdematous and hæmorrhagic infiltrate spreading over abdomen from seat of injection and extending deeply into the thigh muscles, and miliary purpura of stomach. There was no cataract, and the urine was quite clear. Rabbit A showed a large œdematous and hæmorrhagic subcutaneous area over upper thorax, neck, and fore limbs, also a larger hæmorrhage under skin of left cheek. Miliary purpura of stomach and slight purpura of intestine. Liver yellow and mottled. No cataract. Urine quite clear.

The other animals remained well and put on weight.

Dec. 11th: Rat A dead. Post mortem: Purpura of skin particularly marked over skull and cheek. A few spots in pyloric portion of stomach and a few in small intestine. Urine was blood-stained (hæmoglobinuria).

The other animals, which received the heterologous plate sera, remained well and put on weight.

Summary and Conclusions.

1. Data have been recorded on the blood changes in guinea-pigs after inoculation with anti-guinea-pig-plate serum. The early fall in the number of platelets simultaneously with the outburst of skin purpura is found to be a marked feature.

2. Purpura has also been produced in rabbits and rats by inoculation with the respective anti-plate serum. The blood changes in rabbits have been followed with results similar to those obtained in guinea-pigs.

3. Anti-plate serum, in addition to its purpura-producing properties, possesses in common with other cytolytic sera considerable lytic powers, which in all probability contribute largely towards the fatal issue in small animals. Death, however, may occur as the result of extensive hæmorrhages

alone without any obvious lytic changes as evidenced by hæmoglobinuria.

4. Sera obtained by immunisation with red cells or leucocytes do not produce purpura.

5. Purpura is not produced by inoculating animals with heterologous anti-plate sera.

The histology of the purpuric lesions will form the subject of a later communication.



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N^o 12REPORT UPON THE POST-MORTEM EXAMINATION
OF RATS AT IPSWICH¹.

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INTRODUCTION.

ON June 30th, 1911, we proceeded to Ipswich, with instructions from the Local Government Board to assist in an enquiry, the purpose of which was to determine the extent of the plague epizootic amongst rats in the surrounding districts. The share of this work allotted to us consisted in examination, at the Municipal Laboratory, of the animals received, and in selection, for further investigation by the Board's pathologists in London, of material which appeared suggestive of plague. For this purpose, rats presenting features in any degree consistent with the presence of plague were regarded by us as suspicious.

PRELIMINARIES.

The rats were delivered at the laboratory in boxes. These were opened, and the rats taken out and affixed to dissecting boards. Dissections were made so as thoroughly to expose the principal lymphatic glands, thoracic and abdominal viscera. After inspection, rats obviously free from plague and in other respects devoid of interest, were removed to suitable receptacles, pending transference to the destructor. Those of pathological interest, either with regard to the possibility of plague-infection or from other cause, were set apart for further scrutiny.

For purposes of identification and reference, all rats were numbered.

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Rats set aside on the strength of gross post-mortem appearances as possibly infected with plague were submitted to a complete microscopical examination. Films from heart-blood, liver, spleen and lymphatic glands, stained with carbol-thionin blue, were searched for the presence of organisms resembling plague bacilli. After this, the evidence, macroscopic and microscopic, was reviewed, and the rat was either rejected or reserved for cultural and inoculation tests. In the latter case, the liver, spleen and any other portions indicated by the post-mortem characters were packed and sent to London, accompanied by cards describing the pathological features presented by the animal from which they were taken. Cultivations were usually taken from the organs before despatch. Rats which had been so treated were then immersed in strong formalin, and removed separately in tins for destruction. A record was kept of other features of pathological interest.

NUMBER OF RATS, &C. EXAMINED.

Summary of Results.

Sexes and Pregnancies :

Males	7,490
Females	7,342
Pregnant Females	1,213
Average number of Foetuses	9.9

Species :

<i>Mus decumanus</i>	15,167
<i>Mus rattus</i>	165 ¹
Ferrets	2
Moles	3
Hares	3
Stoat	1
Weasel	1
Rabbit	1

Plague infection.

	Killed	Found dead	Total
Rats examined	15,263	69	15,332
Sent up as suspicious, and proved negative	105	11	116
Proved plague infected by inoculation ...	21	14	35

¹ This number includes 134 rats caught on board ships in the port of Ipswich, and 31 caught in and around the docks.

The work was performed by us with the help of two attendants, and we wish to record our appreciation of the valuable and conscientious service rendered by J. Yerrell, our chief assistant.

POST-MORTEM APPEARANCES.

The accompanying tables show diagrammatically the characters of the selected rats, those found upon further enquiry in London to be infected with plague being placed in Table I, whilst particulars regarding those which proved to be negative are set forth in like manner in Table II. A certain number of rats which were sent up and found to be negative are excluded from Table II for reasons hereinafter stated. (See p. 321.) The following paragraphs contain some amplification of these tabular statements.

Plague-Infected Rats.

Lymphatic Glands.—Enlargement of glands was present in most infected rats; three only failing to exhibit this feature. It was confined to the submaxillary region in five instances, whilst four rats showed congestion of these glands with no increase in size. On eight occasions, the lymphatic glands showed general congestion, but no enlargement. In some of the rats the enlarged glands were associated with minute ecchymoses upon the surface or on section, and definite haemorrhages were observed in a few cases. These appearances were accompanied in some instances by a peripheral zone of congested tissues surrounding the gland, and paling off into a less-marked degree of general subcutaneous congestion (2876 and 3430). In one rat (2876) cross section of an enlarged and congested inguinal gland displayed a necrotic centre. In another (15,079) the left pelvic gland was necrotic. Involution forms, resembling those of *B. pestis*, were observed in some cases, upon microscopic examination of smears from enlarged glands (2876, 11,724, 12,013, and 13,663). Bipolar bacilli were demonstrated in 23 cases.

Subcutaneous Congestion. Congestion, distributed universally over the subcutaneous tissues, was seen in 24 cases. In two instances (2064 and 2522) it was described as being confined to the thoracic and axillary regions. In four cases only was it altogether absent. This feature was very generally present to a marked degree in the infected rats obtained during the concluding period of the investigation. In general, the congestion was manifested as a uniform subcutaneous blush due to distension

of the capillaries, but in a few cases venous engorgement was most prominent, dilation of individual venules being obvious.

Haemorrhages.—Small haemorrhages were found in 10 rats, but in no case was this feature conspicuous. Four instances of retroperitoneal haemorrhage, the pelvic glands being invaded in two cases, were observed. The lungs showed minute ecchymoses on two occasions. Effusions of blood into the bronchial glands and mesentery, and into the subcutaneous tissues of the axillary and submaxillary regions were also described.

Pleural Effusion.—In three plague-infected rats there was no effusion into the pleural cavity. In one putrid rat turbid fluid was found; four cases showed blood-stained effusion. In all the remaining cases a clear pleural effusion, usually considerable in amount, was observed.

Liver.—The pathological changes exhibited by the liver varied greatly in degree and in quality, variations in size, colour and consistency, surface and surface markings being observed. In two rats, both somewhat decomposed, the liver was not described as abnormal. Three rats (2064, 5381, and 13,817) presented livers which appeared at first sight to be normal, but scrutiny with a hand lens showed the presence of fine pitting distributed universally over the surface of the organ, indicative, possibly, of fibrotic change. In a fourth animal (13,663) this pitting was accompanied by signs of congestion. Enlargement, with congestion, was recorded on nine occasions. No register was kept of variations in the degree of consistency of the organ, as this depended to a large extent upon its state of preservation. In 17 cases, the liver is said to be mottled. The term “mottling,” as applied to liver changes, is not intended to indicate any specific pathological conditions, but is used to indicate a mottled or marbled appearance of the organ associated with abnormal definition of the liver lobules. This condition was often shown by livers undergoing putrefactive change (*e.g.* 10,848).

Granular livers, in which were present necrotic foci appearing as scattered whitish points, were found in 18 cases. In two instances the liver is described as granular but no obvious areas of necrosis were observed. It was noticed in one rat (8086), which had been found dead, that although the liver showed advanced putrefactive changes, whitish points of necrosis stood out clearly from the discoloured surface. The manner in which these various features—congestion, mottling, and necrotic change—were combined in different cases, is shown in the table; but it may here be stated that the complex to which has been applied the name of “typical pest liver” (*i.e.* an enlarged, pale, mottled

liver covered with numerous necrotic points) was found only in three cases (2876, 5381, and 12,012).

In every animal except one (1264) microscopical examination showed the presence of bipolar bacilli in liver smears, well-marked involution forms being observed in a number of cases. In two livers (2522 and 11,407) bipolar bacilli were seen in relatively small numbers, together with a large variety of putrefactive forms. In Rat No. 8086 referred to above, very large involution forms of the swollen and globular types were observed.

Spleen.—In eight cases the spleen was macroscopically normal. Fourteen rats showed spleens which presented signs of congestion, being dark in colour and firm in texture. Fifteen exhibited various degrees of granular change. Enlarged spleens showing no distinctive pathological change were not regarded as abnormal. In two cases (2876 and 13,817) coarse punctate necrotic foci were observed. In one rat (1030) the spleen showed a white infarction.

Microscopical examination showed the presence of bipolar bacilli in 26 cases, enormous numbers being observed in seven of these. Well-marked involution forms were found in several instances (*e.g.* 8086). Spleen smears from seven rats did not show any organisms, although with one exception (1264) these were found in corresponding preparations from the liver. It appeared that there was little correlation between the degree of gross morbid change observed and the number of bacilli seen on microscopical examination. In some cases (3430 and 14,855) spleens which appeared normal to naked-eye inspection yielded films found to be crowded with organisms. This condition was met with in the case of the liver in two cases (5382 and 13,817).

Heart Blood.—Films prepared from the heart blood showed no organisms in six cases. The type of organism observed in the remainder was similar to that shown by the smears from liver and spleen. The number varied from very few (in 1264) to enormous quantities (in 1694). The small number which was found in 1264 afforded in that case the only microscopical evidence of plague infection, as the smears from liver and spleen showed no organisms.

Oedema, etc.—Pelvic oedema was seen in one case (1769). Another rat (10,848) showed the rare condition of general subcutaneous oedema. In Rat 13,817 the lungs were markedly engorged, and were found upon microscopical examination to contain large numbers of bipolar bacilli.

Negative Rats.

Particulars are shown in Table II of 76 rats selected as possibly plague infected, which, however, were shown by cultural and inoculation experiments to be negative. Some of the salient features of these rats are summarised below. In addition to the negative rats included in Table II 35 rats were sent up for further investigation: 19 of these exhibited abundant pleural effusion containing numerous bipolar bacilli, and are more fully described below; the remaining 16 were found to harbour bipolar bacilli in their accessory sexual glands. In most rats, even those apparently healthy, smears from accessory sexual glands showed large numbers of organisms, which often exhibited bipolar staining and bore a close resemblance to the plague bacillus. Cultural investigation and inoculations carried out in these cases proved the absence of any such infection. As soon as it was ascertained that neither of the conditions was in any way associated with infection suggestive of plague, such animals were regarded as negative.

Lymphatic Glands.—Glandular enlargement was a very common feature, and was particularly well marked in two cases. Bipolar bacilli were found in gland smears from 21 rats, and in some cases organisms resembling plague involution forms were observed. Enlargement of lymphatic glands, particularly those in the submaxillary region, was observed in a great majority of the rats received for examination, but in most cases, when microscopic examination was made, no organisms were found. The pelvic glands also were as a rule conspicuous in the rats examined during the course of this enquiry, although it had previously been observed by one of us who had the opportunity of examining a series of rats in January, that these glands were not usually obvious at that season. In the majority of cases enlargement of the glands was not accompanied by congestion.

Subcutaneous congestion.—Intense and general subcutaneous congestion was only observed twice. Localised congestion was seen in a number of cases. In 22 rats no signs of congestion were observed.

Haemorrhages.—Haemorrhages were described as occurring in 11 cases. The majority of these were strictly localised and were probably traumatic in origin.

Pleural Effusion.—Clear pleural effusion was observed in 39 cases, the amount of fluid being scanty in the majority of these. Effusion described as blood-stained occurred in 39 instances. In several decomposed rats turbid fluid was found in the pleural cavity. These numbers

include 19 rats not included in Table II. In 12 of these the pleural effusion was blood-stained, and in seven instances it was clear or turbid. Microscopical examination of films prepared from such pleural effusions showed the presence of a great variety of organisms, some of which simulated very closely both typical and pleomorphic forms of plague bacilli. Further, the groupings of organisms on some films bore a most striking resemblance to those characteristic of *B. pestis*. These organisms were subsequently regarded as being putrefactive.

Liver.—The livers showed punctate foci of necrosis in 16 cases, in five of which the feature was well marked. In four such rats infection with the Gaertner bacillus was subsequently demonstrated. Microscopically no organisms were found in 26 cases; 50 showed the presence of bipolar bacilli.

Spleen.—Granular changes in the spleen were frequently observed. In three animals the spleen showed necrotic foci. Bipolar bacilli were found in 42 rats.

Heart Blood.—Films prepared from the heart blood showed coccobacilli or bipolar bacilli in 36 rats.

Minor changes in the viscera were observed in a great number of rats which were rejected at once, as well as in those reserved for microscopic examination. Mottling or marbling of the liver due to commencing putrefaction was very common. Minor granular changes in the spleen, overgrowth of submaxillary glands, and effusions of blood into the pleural cavity were noticed with great frequency. No statistical record of such changes was kept, but, in about 600 such rats, microscopic examinations were made. These gave negative results.

OTHER PATHOLOGICAL CONDITIONS.

Gaertner Infection.

In 14 of the cases, where infection with plague was, upon preliminary examination, considered possible, further investigation demonstrated the presence of bacilli of the Gaertner group. The allocation of these organisms was based upon cultural and fermentation reactions.

Five other rats were noticed, which demonstrated so clearly the post-mortem character associated with Gaertner infection, that they were not included among the plague-suspects. Nineteen rats altogether were, therefore, regarded as being infected with this organism. It is probable, however, as the investigation was not directed definitely to

the detection of such cases, that this number does not represent the degree of prevalence of this disease among the rats received.

The most prominent post-mortem features typically associated with Gaertner infection were displayed by the liver and spleen. The former was not as a rule granular, but thickly peppered with white points. It should be noted that although a typical plague-infected liver differs markedly from the appearances presented by the organ in a typical Gaertner infection, in certain cases the post-mortem appearances are of no great assistance in differential diagnosis, and further investigation is needed to determine the nature of the disease under consideration. The spleen, on the other hand, was much more markedly granular than that found in the majority of plague-infected rats. Microscopically the number of organisms seen was usually small, but these exhibited, not infrequently, bipolar staining. Some slight amount of subcutaneous congestion was shown by a number of Gaertner-infected rats.

Trypanosomiasis.

Trypanosomiasis was very common, particularly during the first two months of the investigation. The most general and prominent indication of this infection was blood-stained pleural effusion consisting in many cases of pure blood. Rats exhibiting such a blood-stained effusion, whilst appearing fairly healthy in other respects, might with some degree of certainty be regarded as infected with trypanosomiasis. A continuous series of 2000 rats received in August were investigated particularly with regard to this point. Of these, 145 showed pleural effusion which was decomposed and putrid in many cases, and clear in some others, while in 55 cases the fluid was blood-stained: 45 of these showed the presence of trypanosomes. These parasites were also found in five cases where the pleural effusion was turbid. Incidentally, three rats in this series of 2000 were found to be plague-infected. In these three (2522, 2876, 3346) the effusions were severally turbid, clear, and blood-stained. Other appearances, associated with trypanosomiasis, were congestion of the subcutaneous tissues and lymphatic glands, enlargement of glands, and congestion of the liver. In one case these features were particularly obvious, and the right inguinal gland, particularly, was enlarged and surrounded by a zone of deeply congested tissues. Microscopic examination of this gland showed the presence of many trypanosomes, the agglutination phenomenon being well demonstrated. Large numbers of trypanosomes were found also in the heart

blood. This rat was interesting, as showing that the infection was introduced probably within the area drained by the right inguinal gland. It was also very important, in that the post-mortem appearances presented a picture very closely resembling that associated with plague. Agglutination of trypanosomes was, on another occasion, found in a film prepared from the turbid pleural effusion.

Other Infections.

Suppurative conditions of the lungs occurred with great frequency. This phenomenon is extremely common amongst rats, and has been observed on every occasion when large numbers have been examined. In one series, all stages of the condition were seen, from the earliest, presenting a few small foci containing glairy material, to advanced stages in which the lung tissue is almost entirely replaced by caseous matter, the healthy lung which remains being thereby compressed and displaced and the bony wall of the thorax deformed. Suppurations, general and localised, in the abdominal cavity were not infrequent, and abscesses in lymphatic glands were very common. Osteomyelitis of the hind-limb bones was twice found.

Twenty-five cases of rat-leprosy were observed.

DISCUSSION.

Some facts, observed in the study of both negative and positive cases, may illustrate certain difficulties of preliminary diagnosis, which assume their greatest importance in the investigation of outbreaks of rat-plague occurring in this country.

Infected rats presenting atypical appearances were relatively common, particularly during the early part of the enquiry, at the commencement, that is, of the seasonal epizootic. On the other hand, those found during the last two months of the investigation showed features typical of plague. Broadly speaking, preliminary diagnosis during the first period of two months was usually tentative, while during the second period it was, in the great majority of instances, certain. It must not be assumed, however, that this statement implies that negative cases could always be diagnosed as such without resort to animal experiment. Even in regard to cases in which the diagnosis appeared certain, a caveat must be entered. A study of the characters

Under the heading Macroscopic Appearances, these signs refer to plague-like bacilli, thus :
 + + + = well-marked ; copious.
 + + = intense ; very copious.
 + = not examined.
 ... = not examined.
 + + + = many.
 + + = very many.
 + = some.
 ... = not examined.
 + + + = blood-stained.
 K = killed.
 D = found dead.

Serial No.	Macroscopic appearances										Killed or found dead					
	Glands			Liver			Spleen		Microscopic appearances							
	Enlargement	Congestion	Subcutaneous congestion	Hæmorrhages	Pleural effusion	Necrosis	Mottling	Congestion	Necrosis	Granulation		Congestion	Glands	Liver	Spleen	Heart Blood
1030	+		-	-	-	+	+	-	-	+	-	..	+	-	+	K
1264	-	±	+	-	±	+	+	-	-	+	-	..	+	-	±	K
1691	+	±	+	+	±*	+	+	-	-	+	-	..	+	+	+	D
1691	+	+	+	+	±*	+	+	-	-	+	-	..	+	±	+	D
1769	+	+	+	-	+	+	-	+	-	-	±	..	+	+	+	D
2064	+	+	+	-	±	-	-	-	-	-	-	..	+	+	+	K
2156	±	-	+	-	+	-	+	-	-	-	-	..	+	+	-	K
2522	+	-	±	+	+	-	+	-	-	+	-	-	±	±	±	D
2876	+	-	+	+	+	+	+	+	+	+	+	+	+	±	+	D
3346	+	-	+	+	+	+	+	+	+	-	+	+	+	+	+	D
3430	+	+	+	+	-	-	+	-	-	-	-	+	+	+	+	D
4849	+	+	+	+	+	-	+	+	-	-	+	+	+	+	+	K
5381	+	+	±	+	±	+	+	+	+	±	+	+	+	+	+	K
5382	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	K
7643	+	+	+	-	+	-	-	-	-	+	+	..	+	+	-	D
8086	+	-	+	-	+	+	-	-	-	+	+	..	+	+	+	D
10169	+	+	+	-	±	+	+	+	+	+	+	..	+	+	+	K
10840	+	+	+	-	±	+	+	+	+	+	+	..	+	+	+	K
10848	+	+	+	-	+	+	±	-	-	+	+	..	+	+	+	K
11107	-	+	+	+	+	+	+	+	+	+	+	..	+	+	+	K
11724	+	+	+	+	±	+	+	+	+	+	+	..	+	+	+	K
12012	+	+	+	+	+	+	+	+	+	+	+	..	+	+	+	K
12013	+	+	+	+	+	+	+	+	+	+	+	..	+	+	+	K
12245	+	+	+	+	+	+	+	+	+	+	+	..	+	+	+	K
13663	+	+	+	+	±	-	+	+	+	+	+	..	+	+	+	D
13712	+	+	+	+	+	+	+	+	+	+	+	..	+	+	+	D
13743	+	+	+	+	+	+	+	+	+	+	+	..	+	+	+	D
13817	+	+	+	+	+	+	+	+	+	+	+	..	±	+	+	D
14499	+	+	+	+	+	+	+	+	+	+	+	..	+	+	+	K
14816	+	+	+	+	+	+	+	+	+	+	+	..	+	+	+	K
14854	+	+	+	+	+	+	+	+	+	+	+	..	+	+	+	K
14855	+	+	+	+	+	+	+	+	+	+	+	..	+	+	+	K
14911	+	+	+	+	+	+	+	+	+	+	+	..	+	+	+	K
15079	+	+	+	+	+	+	+	+	+	+	+	..	+	+	+	D
15219	+	+	+	+	+	+	+	+	+	+	±	+	+	+	+	K

TABLE II. *Negative Cases.*

Serial No.	Microscopic appearances										Killed or found dead						
	Glands			Liver		Spleen		Microscopic appearances									
	Enlarge-ment	Conges-tion	Subcu-taneous conges-tion	Hæmorrhages	Pleural effusion	Necrosis	Mottling	Conges-tion	Necrosis	Granu-lation		Conges-tion	Glands	Liver	Spleen	Heart blood	
51	+	-	+	+	+	+	+	+	+	-	+	+	+	-	+	±	K
92	+	-	-	-	-	-	+	-	-	±	-	+	-	-	±	±	K
102	+	-	-	-	-	-	+	-	-	±	-	+	-	-	-	±	K
334	+	-	-	-	+	-	+	-	-	+	-	+	-	-	-	-	K
444	+	-	-	-	-	-	+	-	-	+	-	+	-	-	-	-	K
544	+	-	-	-	-	-	+	-	-	+	-	+	-	-	-	-	K
550	+	-	+	-	+	+	-	-	-	+	-	+	-	-	+	+	K
669	+	-	+	-	+	+	+	+	-	+	-	-	+	+	-	-	K
711	+	-	+	-	+	+	+	-	-	+	-	+	+	-	-	-	K
723	+	+	+	+	+	-	+	-	-	-	-	+	+	+	-	±	K
752	+	+	+	+	-	-	±	-	-	-	-	+	-	+	-	-	K
818	+	+	+	-	+	-	-	-	-	-	-	+	-	+	-	-	K
920	+	+	+	-	+	-	+	-	-	+	-	+	-	+	-	-	K
1444	+	+	+	-	±	±	-	-	-	-	-	-	±	-	-	-	D
2557	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	K
2651	±	-	+	-	-	-	-	-	-	-	-	+	-	-	+	±	K
2952	+	+	+	-	±	-	-	+	-	-	-	-	-	-	-	±	K
2994	-	+	-	-	+	-	+	+	-	-	-	-	+	+	+	±	K
3023	+	-	+	-	+	-	-	+	-	-	-	-	+	+	+	+	K
3030	+	-	+	-	+	+	-	+	-	-	-	+	+	+	+	±	K
3031	+	-	-	+	+	+	-	+	-	-	-	-	+	+	-	±	K
3051	+	-	-	+	+	-	-	-	-	-	-	-	+	+	+	+	K
3052	-	-	-	-	+	-	-	-	-	-	-	-	+	+	+	+	K
3053	-	-	-	-	+	-	-	-	-	-	-	-	+	+	+	+	K
3056	-	-	+	+	+	-	±	-	-	-	-	+	+	-	-	±	K
3281	+	+	-	+	+	-	±	+	-	-	-	-	+	-	-	+	K
4187	+	+	+	+	+	-	±	+	-	-	-	-	+	-	-	±	K
4242	+	+	+	+	+	-	-	-	-	-	-	-	+	-	-	+	D
4374	-	+	+	-	+	-	-	-	-	-	-	+	+	-	-	+	K
4549	+	+	+	-	+	-	-	-	-	-	-	-	±	-	-	±	K
4659	+	+	+	-	+	-	-	-	-	-	-	-	±	-	-	±	D
4667	+	+	+	-	+	-	±	+	-	-	-	-	+	+	-	±	K
4761	+	+	+	+	+	-	±	+	-	-	-	-	+	+	-	±	K
4843	+	+	+	+	+	-	±	+	+	+	+	+	+	+	+	+	K

[illegible]

shown by some rats (4843, 4886, and 10,150), in Table II, will illustrate the danger of making positive diagnoses upon the evidence afforded by post-mortem features, not confirmed by inoculation experiments. Reference, on the other hand, to three cases (1264, 2064, and 11,407), in Table I, will illustrate the necessity of care in the investigation of epizootics where rats presenting such slender evidence of infection may occur. Reports of similar investigations carried out at Hamburg and elsewhere indicate that such atypical plague rats may always be encountered in the investigation of European epizootics.

The individual features of greatest service for diagnosis were subcutaneous congestion, clear pleural effusion, and granular, necrotic changes in the liver. All three were present, usually to a marked degree, in most of the plague-infected rats, but only in 9 rats found to be negative. Pleural effusion, either clear or blood-stained, and congestion were observed in a considerable number of cases. No rat found positive failed to display obviously one or more of these characters. In order of relative value, subcutaneous congestion and pleural effusion would come first, the liver changes being less important, but their collective significance should be considered rather than the individual prominence of each single factor.

Enlargement of lymphatic glands possesses, generally speaking, little weight as primary evidence. At the season in which the enquiry was carried out, pelvic glands were conspicuous in most rats. Sub-maxillary glands were enlarged as a rule. Congestion in regions where this feature was generally not obvious was of some value as a secondary piece of evidence.

In general the facts observed agree with those which emerge from the great bulk of work which has been published upon the question of plague epizootics.

Reference has already been made to the misleading character of gross morbid changes associated in one case with trypanosomiasis. Enlarged and congested glands, subcutaneous congestion, pleural effusion, engorgement of liver and spleen, all features suggestive of plague, were shown, though microscopic examination demonstrated readily the real nature of the infection.

In one case a dual infection of pest and trypanosomiasis was present, and it is difficult to say how far each infection was responsible for the post-mortem appearances observed.

The peppery liver associated with Gaertner infections is a crisply defined pathological entity, but in many cases this organ presented

appearances which caused trouble in diagnosis. Concurrent microscopic and macroscopic evidence gave in most cases clear indication of the true nature of the infection, but atypical livers and spleens showing signs of congestion with minor necrotic and granular changes were in a number of instances reserved for inoculation purposes. The result of inoculations with such material showed the importance of microscopic examination, for negative results were obtained in every case where no bipolar bacilli were found in the films.

Microscopical examination presents, however, a number of fallacies. Bipolar staining is at times shown in intestinal and putrefactive organisms, as well as by certain bacteria, probably of intestinal origin, which are found in the accessory sexual glands of otherwise healthy rats. Putrefactive organisms may also present forms resembling very closely pleomorphic plague bacilli, and assume groupings usually regarded as characteristic of pest. These were best seen in decomposed pleural effusions. Inoculation of such material from 16 cases—not recorded in Table II—showed the fallacious nature of such appearances. As previously mentioned, many rats not infected with plague revealed organisms in the liver and spleen which could not be differentiated from plague bacilli on microscopical examination alone.

In the infected rats generally, plague bacilli were less numerous in blood films than in spleen smears, but in a few cases the reverse held true. In some rats received in October plague bacilli were present in enormous numbers in the blood. In one or two of these, the experiment was tried of cutting off an inch of tail and preparing a film from the cut surface of the stump. Films crowded with plague bacilli were thus obtained. This point has a practical application, indicating that the practice, prevalent amongst those who catch rats, of cutting off the tails is not without danger. The cut stump presents a sharp vertebral point which might readily inflict an inoculation scratch.

CONCLUSIONS.

1. Many plague-infected rats in the earlier period (July and August) of the epizootic presented atypical appearances.
2. The majority of those found in the later period were typical and could usually be diagnosed upon post-mortem examination with a considerable degree of certainty. A number of the negative cases presented difficulty in diagnosis throughout the whole enquiry.

3. Features suggestive of plague infection may be simulated by rats infected with trypanosomiasis or with bacilli of the Gaertner group.

4. The most valuable diagnostic points are the presence of subcutaneous congestion, pleural effusion, and necrotic changes in the liver. These should be considered collectively.

5. Minor granular and necrotic changes in liver and spleen uncorroborated by other suggestive pathological characters, macroscopic and microscopic, are in general not indicative of plague.

6. Major granular and necrotic changes in liver and spleen are very suggestive, but not necessarily indicative, of pest infection.

7. Fallacies in direct microscopic diagnosis are presented by certain intestinal and putrefactive organisms.

TETANUS:

ITS PREVENTION AND TREATMENT BY MEANS OF ANTITETANIC SERUM.

TETANUS cannot be said to be of frequent occurrence under ordinary conditions of life in time of peace, but the outbreak of war is often followed by a comparatively large increase in the number of cases of this disease.

These notes, the fruit of work in a serum laboratory and of a slight cultivation of the literature, were gathered together some months ago without any idea of their appearing in their present form, and I now publish them with the object of calling the attention of those who have not specially studied the question to the value of tetanus antitoxin, which is inestimable when the serum is used properly as a prophylactic. It is to be hoped that at the present time systematic use will be made of this remedy, so that one, at any rate, of the hazards run by those who are offering their all on the battlefield in defence of their country may be reduced to a minimum.

HISTORICAL.

Tetanus, or lockjaw, is a malady common to man and many animals. It was recognized in ancient times, and it has long been known that it was likely to follow the soiling of wounds with earth, dust, and similar material. About the middle of the nineteenth century the impression gained ground that it was due to the production of a poison in the wound, and in 1884 Carle and Rattone reported that they had conveyed the disease by means of pus from a case of tetanus.

In 1885 Nicolaier produced tetanus in mice, guinea-pigs, and rabbits by inoculating them with garden soil, and the pus from the wounds carried on the infection from animal to animal. In the local lesion Nicolaier found a bacillus with a spore at the end, but he did not succeed in obtaining a pure culture.

In 1886 Rosenbach found a similar bacillus in a case of human tetanus, but he also was unsuccessful in his attempts to isolate it. Knut Faber (1889) separated the toxin from mixed cultures by means of filtration through porcelain. Kitasato in 1889 was the first who succeeded in securing a pure culture of this bacillus and in proving that it alone was the causal agent of the disease.

DISTRIBUTION OF THE BACILLUS.

The *Bacillus tetani* is widely distributed in nature, being found in the soil of most cultivated areas, in the streets of cities, and in fact in any place where there is faecal contamination. v. Lingelsheim recalls the examination by Bossano of samples of dust from thirty-eight towns, only twelve of which were found free from tetanus bacilli. Nicolaier found it in twelve out of eighteen samples of earth in the neighbourhood of Göttingen.

Choukévitch (1911) demonstrated its presence in the intestinal contents of two horses out of eighteen, but Lukas (1914) was more successful, as he isolated it from the faeces of sixteen out of seventeen horses. Joseph (1910) examined the excreta of cattle, and came to the conclusion that the *Bacillus tetani* must be considered a normal inhabitant of the intestine of these animals. Römer (1909) also found it frequently in the faeces of adult cattle, but it was not usually present in animals under 2 years of age. Further, tetanus antitoxin was found in the blood of the older animals in more than 50 per cent. of the cases examined. Dolly (1910) found tetanus bacilli frequently in the wads of blank cartridges, and Graser (1910) reports a like result. Rabinowitsch (1907) found the spores of this bacillus in the water in which strawberries sold in Berlin had been washed. It is said to have been found in the mud of the Dead Sea.

This wide distribution of the organism emphasizes the necessity for extreme care in cleansing all wounds, but especially those which have come in contact with street dust, road sweepings, the soil of cultivated fields, garden earth, manure, etc.

Bahuson (cited by Anders and Morgan) stated that during the American Civil War he was put with other prisoners into a shelter that had been used for horses, and that all the wounded placed there who did not die as a direct result of their wounds developed tetanus, and all of them died.

CULTIVATION OF THE BACILLUS.

Under the usual conditions of artificial cultivation in pure culture, the tetanus bacillus will not grow in the presence of oxygen, and therefore it is termed an "anaërobic" organism. But if some nutrient medium be inoculated with the *Bacillus tetani*, and also with some oxygen-loving organism such as the *Bacillus subtilis* (hay bacillus), the absence of oxygen is not so necessary, as the *B. subtilis* will use up the oxygen and produce conditions in which the *B. tetani* can grow. Further, Tarozzi and others have shown that if pieces of animal tissue be placed in a liquid culture medium the bacillus will grow alone in the presence of oxygen, the dead tissue acting as a reducing agent.

In nature we get a combination of these two methods of producing conditions suitable for growth; in the earth we have organic materials to act as reducing agents and the

aërobic organisms to use up oxygen; in contused and dirtied wounds we have the dead tissues and the pyogenic cocci to act in a similar manner.

PRODUCTION OF TETANUS TOXIN.

Tetanus toxin is prepared by growing the tetanus bacillus in nutrient bouillon, of neutral or slightly alkaline reaction, which may or may not contain some reducing agent such as glucose, sodium sulphindigotate, etc. Special flasks filled with such a broth are inoculated with the tetanus bacillus, hydrogen gas is passed through the liquid and the flasks are placed at 37°C. for eight to ten days, when the bouillon is filtered through porcelain to remove the bacilli and spores. The filtrate forms the tetanus toxin. The toxicity of such a filtrate varies very much. A mouse of 15 grams weight might be killed in three to four days by $\frac{1}{50000}$ c.cm., or it might be necessary to inject $\frac{1}{10000}$ c.cm. or even more. The susceptibility of different species of animals to this toxin varies very markedly; thus it is stated that if a certain quantity of toxin will just kill 1 gram of mouse the same quantity will kill—

6 grams of guinea-pig		$\frac{1}{600}$ gram of cat
12 „ of horse		$\frac{1}{1000}$ „ of goose
$\frac{1}{2}$ gram of goat		$\frac{1}{4000}$ „ of pigeon
$\frac{1}{150}$ „ of rabbit		$\frac{1}{30000}$ „ of hen

from which it is evident that the horse is one of the most susceptible of animals.

PRODUCTION OF TETANUS ANTITOXIN.

In 1890 Behring and Kitasato showed that animals could be immunized against the tetanus bacillus and its toxin, and that then their blood had acquired a new property, so that when it was injected in suitable quantity into other animals these were protected against doses of tetanus which otherwise would be invariably fatal. This new property, they thought, was due to the development in the blood of immunized animals of a substance which, as it neutralized the toxin, they called “antitoxin.” When the fluid part of the blood has been separated from the solids it is found that the antitoxin is contained in the plasma or serum. Horses are the animals most frequently used in the production of tetanus antitoxin for therapeutic purposes. As they are so highly susceptible to the toxin the utmost care is required during the process of immunization to avoid the occurrence of accidents. Therefore, at the beginning of the immunization the toxin is not given in the pure state, but only after having been modified by chemicals or by being mixed with antitoxin. Sometimes the toxin and the antitoxin are not mixed, but are given simultaneously in separate parts of the body or the antitoxin is given a few hours before the toxin. Whichever method is used at the commencement, the object is always the same, namely, to produce a basal immunity so that after a time the animal will bear without ill effect the

inoculation of pure toxin. Therefore, as the injections are repeated the amount of modifying agent is gradually decreased until only toxin is given. When the establishment of a basal immunity has been demonstrated the doses are increased more rapidly, and finally 200 c.cm., 400 c.cm., or 1,000 c.cm. of pure toxin are injected at one time. The inoculations are usually given subcutaneously or intramuscularly (though some use the intravenous route) at intervals of five to ten days. After the final dose a rest of one to three weeks is given before the animal is bled, when on an average 8 litres of blood are withdrawn. If considered desirable another bleeding may be taken at the end of a week, and followed by a third, fourth, or fifth at intervals of seven or eight days. The animal then has a rest of three weeks or more before being reimmunized. During a bleeding, as well as during all subsequent processes, every precaution is taken to prevent contamination. When the plasma or serum has been obtained separate from the corpuscles and clot, a small amount of preservative is added, and it is put away in a cool dark place for a time.

As soon as possible after a bleeding the serum is tested to ascertain whether it contains enough antitoxin to render it fit for therapeutic use. This standardization is carried out according to the American method introduced by Rosenau and Anderson (1908), a description of which has been published in *Bulletin* No. 43 of the United States Public Health and Marine Hospital Service and in the *Journal of Hygiene*, January, 1914.*

When required for use the serum is filtered through porcelain and distributed into small bottles. Before being sent out for therapeutic use antitetanic serum is tested—

1. *For absence of toxicity* by animal experiment 10 c.cm. of serum being injected subcutaneously into each of two guinea-pigs of 250 grams weight. The animals must remain well.
2. *For sterility* by cultural experiment aërobically and anaërobically. The media inoculated with the serum must not show any growth.
3. *For non-excess of preservative*, 0.5 c.cm. of serum being injected subcutaneously into a mouse of 15 grams weight. The animal must not show more than temporary symptoms of intoxication.

From the time the serum is first obtained its antitoxin content begins to diminish. This diminution takes place most rapidly at first; later the serum appears to reach a condition of comparatively stable equilibrium, when the deterioration proceeds much more slowly. To compensate for this unavoidable loss there is usually put into each bottle an amount of antitoxin in excess of the quantity stated to be present, sufficient to make it probable that the bottle will, at the end of two years, contain the amount of

* The Lister Institute serum is thus standardized.

antitoxin stated on the label, provided it is stored in a dark cool place—preferably in an ice-chest.

PROPHYLACTIC USE OF TETANUS ANTITOXIN.

Tetanus antitoxin may be used (1) prophylactically, (2) curatively.

As a prophylactic tetanus antitoxin yields excellent results.

Animal experiment has proved:

That if a fatal dose of tetanus toxin be mixed together with a suitable amount of tetanus antitoxin, the mixture can be injected into animals without giving rise to any ill effect whatever.

That the effect of a fatal dose of toxin can be completely inhibited by the injection of antitoxin twenty-four hours previously.

That a fatal result can be prevented by injecting a suitable dose of serum within a short time of the injection of the toxin.

From these results it would naturally be expected that tetanus antitoxin would furnish good results when used as a prophylactic. Such has been the case.

Vaillard (1912, p. 224) refers to the practice of eight veterinary surgeons from 1898 to 1906. In that period they inoculated 13,124 animals after operations or accidental wounds, and not a single case of tetanus occurred among them. During the same time two veterinary surgeons alone saw 139 cases of tetanus among animals which did not receive the treatment. If the figures of Nocard and Labat are added, we get 16,917 animals which had a prophylactic injection, and among them one single horse had tetanus—in this case the antitoxin was given five days after the wound, and the attack was very benign.

Huguier (1909) used to give two injections of 10 c.cm. each of antitetanic serum, but for some years has been content to give one, and, although he operated in conditions under which infection was most possible, he has not had one case of tetanus among horses thus treated prophylactically.

Dieudonné (1909) records 1,009 operations for castration or hernia, with no case of tetanus, although only a single injection of serum was given. During the same period he observed 87 cases of tetanus among horses operated on or wounded which had not had a prophylactic injection.

Parker Hitchens (1910) has proved experimentally that a dose of 300 U.S.A. units of tetanus antitoxin will protect a horse against an infection fatal to the control animals.

Mohler and Eichhorn (1911) found experimentally that as regards an infection fatal to the control—

300 U.S.A. units given 48 hours after infection = no symptoms of tetanus.

500 U.S.A. units given 72 hours after infection = no symptoms of tetanus.

700 U.S.A. units given 96 hours after infection = no symptoms of tetanus.

Whereas—

- 250 U.S.A. units given 48 hours after infection = local tetanus. Recovery.
 400 U.S.A. units given 96 hours after infection = local tetanus. Recovery.

They conclude "that 500 U.S.A. units is a sufficient dose of antitoxin for use as a prophylactic, even in cases where the infection has occurred four days prior to the injection of antitoxin."

The experience of veterinary surgeons is therefore all in favour of the value of the prophylactic use of antitetanic serum. The results of its use for a similar purpose in the human subject are scarcely less decisive.

Vaillard (1912) cites the experience of Biron and Pied. These surgeons did not use serum from 1895 to 1902, and had during those years 11 cases of tetanus, which were all fatal. In 1903 they began to use serum systematically in every case in which there was a chance of infection with tetanus. From 1903 to 1910 there was not a single case of tetanus in their practice, but during this period they received into their wards 4 cases of tetanus from outside; 3 cases of tetanus were taken into other wards of the same hospital, and there were 2 deaths from tetanus in the town. Vaillard refers also to cases of tetanus following wounds received during the celebrations on July 4th (Independence Day) in America. In 1903 there were 415 cases of tetanus. Then the prophylactic treatment was strongly urged and began to be made use of.

In 1904 there were	...	...	105 cases.
In 1905 " "	...	...	104 "
In 1906 " "	...	...	89 "
In 1907 " "	...	...	73 "

These figures suggest that, as the years passed and the beneficial results were recognized, more and more attention was devoted to prophylactic serum treatment.

Confirmatory evidence has also been brought forward by Brandenstein (1908); Bockenheimer (1909); Solieri (1910), thirty-five cases with one death; Graser (1910), no case of tetanus in eight years among patients treated prophylactically; Simon (1913, p. 236), citing Liell's analysis of 350 cases of July 4th wounds and tetanus, shows a mortality of 98 per cent.; only seven recovered and of these five had prophylactic treatment. In 1907 the editor of the *Journal of the American Medical Association* summed up the case as follows:

A fairly careful scrutiny of the American literature for the past five years has not brought to light a single report of the development of tetanus in a person who received a timely prophylactic dose of tetanus antitoxin.

We thus see that the inestimable value of tetanus antitoxin when properly used as a prophylactic is beyond question.

In connexion with the words "properly used" in the previous sentence it is important to remember that

antitetanic serum is an antitoxic and not a bactericidal serum. It has no power to inhibit the growth of the bacillus. All antitoxin can do is to neutralize the toxin produced. A certain amount (x) of serum can neutralize a certain amount (y) of toxin and no more. Any further toxin produced can exercise its deleterious effect uninfluenced by the serum. Thus if an animal has been infected by tetanus and has received a prophylactic dose of serum it is protected against the toxin produced until the neutralizing power of the serum has been exhausted. When that stage is reached, then, as Vaillard puts it, if more toxin is elaborated one of three things must happen, either:

1. More antitoxin must be given; or
2. The animal must produce its own antitoxin; or
3. The animal must become intoxicated.

Further (cf. Tarozzi *supra*), the tetanus bacillus can multiply and produce toxin in blood clots, or in necrotic tissue, especially in the depths of a wound; and as there is some evidence suggesting that a sharp fall may occur in the antitoxin content of the blood about one week after an injection of antitoxin, it is advisable to repeat the injections weekly as long as there is any necrotic tissue in a wound. Again, there may be necrotic tissue in a wound several days old, or a large amount of dirt may be ground into the tissue—for example, run-over cases—and it may be decided to operate and thoroughly clean up such a wound. In this case it is advisable to give a prophylactic dose of antitoxin intramuscularly some hours before the operation, as acute tetanus has occurred within twenty-four hours of such operation, and has in all probability been due to the rapid absorption, by the fresh tissues laid bare by the operation, of a large dose of tetanus toxin which had been elaborated in the necrotic tissue, or was present in the dirt with which the wound was soiled.

Dose.

The amount of serum which should be given at each injection depends entirely upon the number of units in each cubic centimetre of the serum. We have seen above that for the horse, which is the animal most susceptible to tetanus poison, the Americans have experimentally demonstrated the dose to be 300 to 500 U.S.A. units; but in the case of man we have no such evidence. v. Behring recommends 10 to 20 units. These units are German units, and one of them is equal to about 40 U.S.A. units. The dose would then be 400 to 800 U.S.A. units.

Vaillard mentions 10 c.cm. as the dose for superficial easily-cleaned wounds, not grossly soiled; 10 c.cm. of the tetanus antitoxin sold by the Pasteur Institute usually contains from 500 to 1,000 U.S.A. units.

Tizzoni considers that 200,000 of his own units is an ordinary prophylactic dose. I have only examined one

sample of Tizzoni's serum.* The bottle contained 5 c.cm. = 200,000 units Tizzoni = 125 units U.S.A.

Park and Williams (p. 243) say: "It is the custom at many dispensaries in New York City and elsewhere to immunize all Fourth of July wounds by injecting 1,000 units. None of these have ever developed tetanus."

From this last statement we would conclude that 1,000 U.S.A. units is an ample prophylactic dose, as it has always prevented tetanus, and that it is quite possible a smaller dose would answer the purpose. This apparently has been found to be the case by von Behring, Vaillard, and Tizzoni.

Where there is plenty of serum at the disposal of the surgeon there is no need to take thought about the size of the dose, and the ample dose of 1,000 to 1,500 units may be given with the knowledge that it is better to give too much than too little. But when, as may easily happen during war, there is only a limited amount of serum available, then the question of the smallest protective dose becomes important. This may be put down as about 500 U.S.A. units for a simple uncomplicated case, and as the amount of soiling of the wound and contusion of the tissues increases so should the amount of antitoxin be increased.

The interval of time between the infliction of the injury and the injection of serum also has to be taken into consideration when fixing the dose. Dönitz (1897) showed experimentally in rabbits that if an hour were allowed to elapse between the injection of the toxin and that of the serum it required forty times the neutralizing dose (*in vitro*) of serum to save the life of the animal. Studying the same question recently on guinea-pigs I found that while $\frac{1}{10}$ U.S.A. unit of antitoxin neutralized 100 minimal lethal doses (M.L.D.) of tetanus toxin *in vitro* it required 1 unit of antitoxin to neutralize 1 M.L.D. when given subcutaneously and simultaneously but in different parts of the body, but 3 units did not save life when given four hours after the toxin. When the serum was given twenty-four hours after 1 M.L.D. of toxin it required 2,000 units of antitoxin to save life, though even this amount did not prevent the onset of the disease. In these animals 1 M.L.D. almost always caused the appearance of slight signs of tetanus at the end of twenty-four hours after the injection of toxin.

It is obvious, then, that the earlier the prophylactic dose is given the more certain will be its effect in preventing tetanus, the smaller within limits will be the dose, and the smaller the cost of treatment. This last item may seem insignificant, and it is so when it is only a question of the prophylactic use of antitoxin; but it will be found to loom large in the picture when it is necessary to consider the value of tetanus antitoxin from a curative point of view.

* Cf. *Journal of Hygiene*, January, 1914.

CURATIVE USE.

"Premonitory" Symptoms.—In all diseases the earlier the treatment is commenced the greater the chances of a favourable result. If we could recognize tetanus in a stage as early as we can diphtheria, it would no doubt be possible to obtain just as good results from serum treatment. But tetanus, unlike diphtheria, has no characteristic lesion which appears early in the attack and gives an indication of the nature of the disease. As it has been aptly put by Dr. C. J. Martin, the diagnostic membrane in diphtheria corresponds to the dirt or other foreign body carrying the infection of tetanus, and so, just as we give diphtheria antitoxin when we see such a membrane, so ought we to give tetanus antitoxin when we see any such infecting material in a wound. When the symptoms of tetanus are distinct it means usually that the disease has made very considerable headway, and has reached that stage which in diphtheria would be looked upon by some as beyond the reach of the beneficial influence of antitoxin.

This explains to some extent the uncertain results which follow the use of antitoxin as a curative agent, and it also raises the question whether there are any "premonitory" symptoms the presence of which might rouse a suspicion that a case was one of commencing tetanus. A study of some of the literature of tetanus has shown that commencing tetanus has been mistaken for "colds," muscular rheumatism, stiff neck, sore throat, influenza, mumps, etc., and it has even been suggested that many cases which have been diagnosed and treated successfully as cases of the above diseases have in reality been slight attacks of tetanus. With the object of directing attention to what may prove to be "premonitory symptoms," K. Evler (1910) relates 13 cases of tetanus with 2 deaths, which came under his personal observation, he himself being one of them. He describes these "premonitory" symptoms as being very varied and changeable.

A day or so after the infection there may be general restlessness, changing suddenly to a desire to rest.

Sleeplessness with distressing dreams, and it may be nightly delirium.

Difficulty in micturition due to spasm of the sphincter vesicae, which may last from a few minutes to half an hour.

Temporary giddiness, violent headache, excessive yawning.

The facial appearance changes and the patient looks anxious, though there is no risus sardonicus yet.

There may be trembling of the tongue which is put out to one side.

There is often a profuse sweating, and darting pains in various parts may occur.

The patient may have a feeling of chilliness, and there may be some swelling, without redness locally, of the in-

jured member and throbbing of its arteries notwithstanding that the limb is raised.

Slight jerking may follow pressure on the flexor tendons, and these muscles may be noticed to be in a condition of increased irritability.*

If symptoms such as these are present with a history of possible infection with the tetanus bacillus Evler considers that the administration of serum is justified. The later symptoms include:

Increased flow of saliva.

Reflex cramps of oesophagus.

Ocular symptoms; for example, nystagmus, strabismus.

Ear trouble, and spasmodic cough.

Tremors and clonic spasms which are not painful and which may not attract attention.

Pain persisting after muscular contraction induced by effort.

Swollen and reddened lymphatics, enlarged glands and tenderness in the infected region.

Evler infected himself during an operation and early symptoms developed within twenty-four hours. He presented nearly all of the symptoms mentioned. Sero-therapy was commenced on the fifth day. Nutrient enemata became necessary on the seventeenth and eighteenth days, but on the nineteenth the teeth could be opened a little and recovery then progressed satisfactorily.

Evler's paper directed my attention to these "premonitory" symptoms, and since reading it I have carefully read through a considerable number of cases of tetanus described in the literature, and I have found that practically all the symptoms mentioned by Evler have been noticed as being present early in the disease. All have not been observed in the same patient, but usually the one or two present have been sufficiently prominent to force themselves upon the notice of the physician, and, if he had read Evler's paper, to remind him that such a disease as tetanus might be before him.

If tetanus be studied from the standpoint of these "premonitory" symptoms, there is reason to hope that as our knowledge of them increases so will our power of combating the disease be increased by our intervention earlier in an attack.

Curative Value.

The results following the use of antitoxin as a curative agent have been up to the present for the most part unsatisfactory. The reasons given to account for this are that the disease is usually diagnosed only when it has made considerable progress, and that the toxin is absorbed and travels along paths where it is beyond the influence of antitoxin.

In most cases the tetanus bacilli remain and multiply at the site of infection, and the toxin there produced is

* This condition of increased irritability of the flexor muscles has been noticed to occur in horses quite early in an attack of tetanus.

absorbed and carried to the central nervous system. As regards the path by which it travels, numerous observers have corroborated the findings of Meyer and Ransom that tetanus toxin is chiefly absorbed from the infected wound along the motor nerves of that region, and passes by direct extension into and through the spinal cord, but it may also pass into the circulation. It is also thought that in the nerves the toxin passes along the axis cylinder. Some observers, however, do not agree entirely with these conclusions.

Cernovodeanu and Henri (1907) have stated that if the vessels and muscles of a limb be tied while the nerves remain intact, a large amount of tetanus toxin may be injected without the production of tetanus, and they therefore conclude that, while some toxin is absorbed by the nerves, the larger part passes along the lymphatics and blood vessels.

Field (cited by Park and Williams) is of opinion that the toxin does not pass along the axis cylinder but along the lymphatics of the nerves.

Permin (1912-1913), from a critical analysis of the literature and from the results of a large number of experiments performed by himself, concludes that part of the toxin is taken up by the peripheral muscle nerves of the site of infection and passes along the axis cylinder to the spinal cord, and part passes into the lymph and blood and gaining the general circulation may pass directly to the ganglion cells of the cord or indirectly by means of the peripheral nerves.

There is, then, no unanimity of opinion as to the special road taken by the toxin, but what we know is that somehow the toxin passes along the nerves comparatively rapidly, but the amount found in the cerebro-spinal fluid is not great—on several occasions 1 to 2 c.cm. of this fluid have contained a fatal dose for a mouse but not more toxin than this.

The holding of the opinion that the toxin is absorbed by the nerve endings and passes along the axis cylinder—that is, in a situation which is beyond the reach of antitoxin in the blood—renders the curative treatment of declared tetanus practically hopeless, in the opinion of many clinicians. Antitoxin does not appear to penetrate readily into the substance of the peripheral nerves or of the central nervous system, and therefore all it can do is to neutralize only the toxin which has not been absorbed, or which is in the blood stream. (After huge doses of antitoxin, given intravenously, only traces of it can be found in the cerebro-spinal fluid.) So a cure can result from the employment of antitoxin only so long as a fatal dose of toxin has not been absorbed. Many cases are on record, and many experiments which seem to confirm this view.* The experiments of McClintock and Hutchings (1913) will serve to illustrate this

* Note.—For a most excellent review of the literature of this subject, cf. Permin, 1913.

point. They took sheep with long tails and inserted into the tip of the tail a splinter of wood which had been infected with tetanus spores free from toxin. The first symptoms of tetanus occurred six to eight days later, when the tail was at once amputated, 20 cm. being removed, and 4,500 U.S.A. units of tetanus antitoxin were given intravenously and repeated daily. Every animal but one died and the symptoms did not seem to be influenced by the serum. The amount of toxin in the blood, as proved by animal experiment, was at its maximum between the fifth and seventh day—2 c.cm. of blood containing a fatal dose for a guinea-pig of 350 grams weight. No toxin was found in 2 c.cm. of the blood twenty-four hours after the injection of serum.

From these results it has been concluded that as amputation of the tail had no effect upon the progress of the disease, and as there was toxin in 2 c.cm. of the blood before but not twenty-four hours after the commencement of serum treatment, therefore by the time that the first signs of tetanus appeared a fatal dose of toxin either had been absorbed and fixed, or if not completely fixed was in a situation where the antitoxin could not reach it to prevent complete fixation. This being the case, all that can be expected from serum treatment is the prevention of further absorption of toxin by blocking all the paths leading from the infected area—the nerve routes by injection into the nerves of the region affected and the vascular route by large injections into a vein—and all this merely in the hope that a fatal dose of toxin has not already been absorbed.

It is not certain, however, that these views are correct. They may be based on a false premiss, namely, that antitoxin must penetrate into the cell before it can neutralize the toxin attached to the cell.

But the recent work of Kraus and Amiradzibi (1910) suggests that, contrary to the usual acceptance, a toxin must pass out of a poisoned cell before it can be neutralized by its antitoxin, as the latter cannot penetrate into the cell; and further, that the rate of diffusion of the toxin out of the cell is accelerated by the presence of antitoxin in the surrounding fluid.

Moreover, von Graff and Menschikoff (1912) have shown experimentally that it is possible to extract tetanus toxin from liver cells by means of antitoxin. They allowed the cells to remain in contact with a solution of toxin during one hour at 37° C. Then they removed the cells and washed them free from surrounding toxin. Some of these washed cells were injected into mice and caused tetanus. The remainder were soaked in a solution of tetanus antitoxin for one hour at 37° C., and then removed from the serum, washed free from surrounding antitoxin and injected into mice, which remained quite well. Control experiments showed that while toxin could pass into the cell and be so firmly fixed that several washings would not remove it, serum, on the other hand, could not pass into the cell in any demonstrable quantity. It is justifiable, therefore, to

presume that the toxin was extracted from the cells by the surrounding antitoxin, and that it is advisable to have as high a concentration of antitoxin as possible in the fluids surrounding poisoned cells.

This view of the action of the antitoxin gives more encouragement to the physician to persevere vigorously with serum treatment. It emphasizes the advisability of employing very large doses of serum so as to saturate the system with antitoxin, and thus not only neutralize any toxin which may be passing from the focus of infection into the blood, but also to extract toxin from cells into which it has already passed.

Such treatment, suggested by the results of experimental research, is practically identical with that employed by many physicians to-day as the outcome of years of bedside observation.

Ernest E. Irons (1912) analysed the 252 cases collected by Anders and Morgan* and classified them. The incubation periods of the fatal and non-fatal cases showed practically the same relation. His figures are :

				Mortality.
Total number of cases	...	252	...	71%
Cases treated without serum	...	142	...	77%
Cases treated with serum...	...	112	...	63%
Cases treated with small amounts of serum	...	71	} 112	70.4%
Cases treated with large amounts (100 c.cm. or more) of serum	...	41		
				51.0%

If we compare the mortality of all the cases treated with serum with that of the cases treated without we must allow that the use of serum brings little if any advantage. But if we compare the mortality in cases treated *with large amounts of serum* with the mortality of the cases treated *without serum*, then the beneficial results following such use of serum become manifest.

W. H. Park (1910) says that when a large intravenous dose is given within a few hours of the onset of the symptoms the effect is marked. More than 50 per cent. of the patients so treated recovered, "some of whom I feel perfectly sure would have died without the serum." At the first sign of actual tetanus 10,000 to 20,000 U.S.A. units should be given intravenously, and followed every twelve hours by further injections. The later injections may be given subcutaneously if the intravenous route becomes difficult. Torres (1912), after an experience of 110 cases of acute tetanus in eight years with a mortality of 32.7 per cent., gives an intravenous injection of 120 c.cm. of serum as the rule. He repeats it twice, or even oftener, in the first twenty-four hours if necessary, and then gives 100 c.cm. daily if the symptoms are still present.

Penna (1913) also gives injections of 100 c.cm. intravenously once or twice in the twenty-four hours, and keeps it up for several days *even though the patient may be improving*.

* *Journ. Amer. Med. Association*, July 29th, 1905, p. 314.

Van der Bogert (1913) cites:

1. Strock (1907) who treated his cases of tetanus during six years with small doses of serum and had a mortality of 100 per cent. Then he treated 5 cases with very large doses and the mortality was 20 per cent.

2. The 3 cases of Young (1912) which appeared hopeless, but recovered—a boy had 150,000 U.S.A. units, a man had 220,000 U.S.A. units, and a girl of 14 years had 587,000 U.S.A. units.

Many other similar records could be quoted, if space permitted, to show that among those who not infrequently meet with cases of tetanus the tendency of late years has been to use much larger doses of antitoxin than formerly, and that the use of these large doses seems to be followed by more uniformly beneficial results than were observed in former years when only small doses were employed.

Modes of Administration.

Besides the size of the dose the mode of administration of the serum has an influence upon the result. Serum may be given—

1. Intracerebrally.
2. Subarachnoidally.
3. Intraneurally.
4. Intravenously.
5. Intramuscularly.
6. Subcutaneously.

1. Intracerebral injections have given very good results in animals and in several cases in man also, but the risk of permanent injury to the nervous system has prevented this method coming into general use. Ballance (1914), however, suggests that the introduction of serum into the lateral ventricle might be used in tetanus, as he thinks that with reasonable care the operation as a whole is free from danger.

3. Intraneural injections were strongly recommended by Rogers (1905), but they are not free from risk, and are seldom used.

4, 5, and 6. As regards the intravenous, intramuscular, and subcutaneous route the final result is the same in all three. The only difference is in the time taken for the antitoxin to be absorbed and distributed throughout the body. In the case of the intravenous method it is a question of a few minutes; of the intramuscular, of a few hours; while after subcutaneous injections it is one to two days before the maximum concentration of antitoxin in the blood is reached. There remains the subarachnoid route to consider.

Descos and Barthélemy (1902) experimentally showed that intraspinal injections gave the best result.

Von Graff (1912) carried out a number of experiments in the Serotherapeutic Institute in Vienna, and came to the conclusion that this route was the most effective, and that next to it came the intravenous.

Permin (loc. cit.) considers that intraspinal are just as good as intraneural, and are better than intravenous, inoculations.

W. H. Park (1914, personal communication) has treated 6 successive cases of tetanus by intraspinal injections, and all have recovered.

Hofman (1907) states that of 13 cases which had serum beneath the skin, 53.8 per cent. died, whereas of 16 cases which had serum subdurally only 12.5 per cent. died, and these 16 cases included a number of extremely severe cases with brief incubation periods. He recommends that the intraspinal injections should be given every two to three days and the subcutaneous daily. Graser (1910) also advises serum by intralumbar injection every two to three days and daily beneath the skin. Gobiet (1904), Neugebauer (1905), Jerie (1908), and Buffagni (1910) and others speak of the good effect of intraspinal injections of antitoxin, and Buffagni says:

1. After the first injection one observes an increase in the symptoms, reaching a maximum in forty-five hours, and then passing off and giving place to improvement.

2. The organization of the patient reacts to each injection of tetanus antitoxin by a rise in temperature of 1.5° to $2^{\circ}\text{C}.$, which passes off in ten to twelve hours.

3. After each injection more or less distinct improvement of all conditions occurs, and especially of the tetanic attacks.*

The method of procedure in the serum treatment of a case of tetanus would then be somewhat as follows:

1. The injection beneath the arachnoid, or in very severe cases even (Ballance, 1914) into the lateral ventricle, of as large a dose of antitoxin as may be conveniently given—for example, 3,000 to 8,000 U.S.A. units.

2. The injection into a vein of a large dose—9,000 to 16,000 U.S.A. units—with the object of flooding the system with antitoxin as soon as possible.

3. The injection of antitoxin intramuscularly, and

4. The repeated injection of antitoxin subcutaneously—both with a view of keeping up the concentration in the blood.

Clinical experience both in man and horse has shown that it is absolutely necessary to continue the injections, even though improvement has apparently set in, as otherwise the symptoms are very liable to return—in some cases with greater intensity than at first.

"IDIOPATHIC" TETANUS.

The absence of any history of injury sometimes makes the diagnosis of tetanus at first very difficult. In the past such cases were classed as rheumatic or idiopathic tetanus, but we know now that for tetanus to declare itself the tetanus bacillus must somehow have gained entry into the body.

It has been shown experimentally that tetanus spores free from toxin or foreign material may be injected into animals without causing tetanus, and that they can

*These observations of Buffagni are very like those made by some physicians with regard to intraspinal injections of antimeningococcal serum.

become disseminated in the body and remain latent for a time. Then if suitable conditions arise, such as the formation of a necrotic area in the tissues, such latent spores may germinate and give rise to tetanus.

Canfora (1907) infected animals subcutaneously with tetanus bacilli and found that they spread all over the body. He also injected toxin-free tetanus spores and found them in the blood twelve hours later. They may remain in the blood for ten to fifteen days, during which time very slight injuries are sufficient to set up tetanus. Later, the spores became localized in the liver, spleen, lungs, kidneys, lymph glands, and marrow, where they may remain latent for some months.

Seiple (1911) came to the conclusion that an injection of a solution of quinine might cause enough destruction of tissue to produce conditions favourable for the germination of spores.

It behoves us, therefore, to bear tetanus in mind, even though there be no history of injury, whenever we meet with symptoms similar to the premonitory symptoms mentioned by Erler and found to be present in so many cases of this disease.

PRESERVATIVES IN SERUM.

Most serums have a small amount of preservative (phenol, trikresol, chloroform) added to them, and when doses such as 120 c.cm. are given at one time the presence of preservatives requires consideration and raises the question as to how much preservative can be given without ill effect.

Bacelli (1911), who strongly recommends that tetanus should be treated with subcutaneous injections of carbolic acid, states that not less than 1 gram should be given daily; J. H. Haberman (1913) in a case of hydrophobia gave $22\frac{1}{2}$ grains of phenol subcutaneously in twelve hours without any evidence, either locally or generally, of any deleterious effects; Natoli (1899) injected 5.25 grams of phenol subcutaneously in eighteen days; and Ascoli (1898) gave 75 c.cm. of a 3 per cent. solution without any subsequent toxic symptoms.

The probability of ill effects being due to the preservatives in serum when administered intrathecally has been fully discussed and negated by Flexner in the case of automeningococcic serum. It may, then, be safely assumed that the amount of phenol put into serum is not likely to have any serious consequences.

CONCLUSIONS.

Tetanus may be cured by the administration of anti-toxin, provided that the serum treatment is begun early in the attack and is pursued vigorously and continuously. Unfortunately in the present state of our knowledge a diagnosis is as a rule made only when the disease is in a stage so advanced as to make the results of treat-

ment uncertain, even though very large doses of anti-toxin are used. These large doses are costly, and place the serum treatment of tetanus practically out of the reach of those with shallow purses—unless, of course, the serum be provided by the State. But in the prophylactic use of serum there is within the reach of every one a means of checking the disease. It has been proved beyond the shadow of a doubt that, wherever the prophylactic use of antitoxin has been carried out systematically, tetanus may be said to have disappeared. Tetanus should therefore be looked upon as a preventable disease, and, when one realizes this, there flashes across the mind the memorable question, "If preventable, why not prevented?"

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LXXIII. FURTHER EXPERIMENTS ON VACCINATION AGAINST A BODY-STRAIN OF PLAGUE.

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I FIRST recognised that a difference as regards antigenic properties existed between a body-strain and a virulent agar strain of plague from the fact that in order to vaccinate against the former it was necessary to introduce serum proteins as a source of nutriment for the bacilli which were destined for the preparation of the antigen.

In the report (this *Journal*, Plague Supplement III, Jan. 14, 1914, p. 409), "On the influence of cultivation in serum-containing media upon the virulence and immunising properties of the Plague Bacillus," the method of adding heated horse serum to the nutrient agar upon which the organisms from which the antigen was extracted were grown, was shown to yield a vaccine which in doses of 0.02 mg. protected 75 % of rats against a virulent body-strain. It has been attempted to improve upon these figures by varying the method of preparation. The procedure consisted in (1) varying the source (strain) of the organisms, (2) varying the medium upon which these were grown.

The following four methods were employed:

Method 1. The source of the organisms was a virulent agar strain propagated in the laboratory during several years. This was grown in 10 % serum broth for 24 hours. This culture was used to inoculate Roux flasks containing nutrient agar, the surface of which had been flooded with horse serum, which had been heated for half an hour at 55° C.

Method 2. The source of the organisms was as in Method 1. They were grown as before in serum broth. The surface of the agar was in this case, however, flooded with a heated horse serum extract of ox spleen.

Method 3. The source of the organisms was the spleen of a rat dead of plague. They were grown in fresh horse serum and inoculated on to the surface of agar flooded with a fresh horse serum extract of ox spleen.

Method 4. In this method the source of the organisms was, as in the last method, the spleen of a rat dead of plague. These were grown in fresh rat serum and inoculated on to the surface of agar flooded with fresh rat serum extract of fresh rat spleen.

Four antigens were prepared from the organisms grown in these various media, which will be seen to form a graduated series in which the conditions obtaining in the body of a living rat are gradually approached.

The growth was in each case killed by chloroform and swept off in salt solution. From the thick emulsion of organisms thus obtained a paste was thrown down in the centrifuge. This paste was then mixed with twice its weight of anhydrous sulphate of soda. An appropriate amount of water was added to the dry powder resulting, and the sulphate of soda removed by filtration in the form of a saturated solution at 37° C. The bodies of the bacilli remaining on the filter were extracted in distilled water.

The vaccinating power of the four antigens was then tested against a body-strain of plague obtained from the spleen of a rat dead of plague. A dose of three millions of this strain killed 90 % of uninoculated rats. The original source of this strain was a virulent agar strain which had been passed through rats twice.

The results of this experiment are shown in the following table :

Antigen No.	Strain	Culture	Agar flooded with	No. of Rats inoculated	Percentage which survived the inoculation
1	Agar	Serum broth	Heated horse serum	47	57
2	Agar	Serum broth	Serum spleen extract	39	74
3	Spleen	Fresh horse serum	Serum spleen extract	41	75
4	Spleen	Fresh rat serum	Fresh rat serum spleen extract	43	84

From these results I conclude that as the conditions in the culture medium that is used for the propagation of the organisms that are to afford an antigen approach those that obtain in the body of the living animal, so the efficiency of the antigen against a body-strain of plague is increased.

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LXXIV. IMMUNISATION BY PSEUDOTUBERCLE.

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IN this *Journal* (Vol. XII. Dec. 1912, p. 350) I concluded from the results of experiments that it was possible to immunise the guinea-pig against plague by means of killed cultures of the pseudotubercle bacillus, but that it was very difficult to immunise the rat by the same method. Since the publication of these results I have discovered the important distinction as regards antigenic properties between a culture of the plague bacillus propagated on broth peptone media and on serum or in the body, respectively.

In view of this new knowledge it became necessary to repeat these earlier experiments, using a serum strain of the plague bacillus to test the immunity conferred by the pseudotubercle bacillus.

For the purpose of the tests which follow, a culture was employed that for six months had been grown continuously in the body of the living rat. When required for testing it was isolated on fresh blood agar and grown in serum broth. A dose of three million such organisms constituted the test dose.

The vaccines were prepared in the same manner as before, the pseudotubercle bacillus being grown on agar, killed by chloroform and suspended in salt solution. The vaccinating dose was ten thousand million (approximately the number present on a 24 hours' agar slope), which was the dose of organisms previously employed.

With this vaccine 26 rats were inoculated. One week after the vaccination the rats received the standard dose of living virulent plague (body-strain).

Six rats survived the test.

Similarly 28 guinea-pigs were vaccinated and tested for immunity in exactly the same way.

Four guinea-pigs survived the test.

This result is in striking contrast to that previously obtained in which no difficulty was experienced in immunising guinea-pigs against a virulent agar strain of plague.

The fundamental difference which exists between the agar strain and the serum (body) strain is emphasised in these experiments.

Similar series of rats and guinea-pigs were vaccinated, using the nucleoprotein extract as in the former series of experiments. In these series also no immunity followed the treatment.

The original statement as to the possibility of vaccinating against virulent plague by means of the pseudotubercle bacillus must now be modified to the following:

It is possible to immunise guinea-pigs against a virulent strain of plague propagated upon broth peptone media, by means of the pseudotubercle bacillus, but not possible to immunise either rats or guinea-pigs against the same strain of plague taken from the body and grown in serum-containing media, either by the whole bacillus or by means of the nucleoprotein extract obtained from it.

LXXV. IMMUNISATION BY LIVING AVIRULENT CULTURES (STRONG).

BY SYDNEY ROWLAND, M.A., M.R.C.S.,
Of the Lister Institute, London.

IMMUNISATION by living avirulent cultures has been advocated and actually carried out by Strong (*Philippine Journal of Science*, Vol. II. No. 3, June 1907).

By his courtesy I have had the opportunity of testing the immunising power of his culture on white rats. It proved quite avirulent both to guinea-pigs and rats, and all attempts to raise its virulence failed. Strong found that he was able to immunise guinea-pigs by the previous subcutaneous inoculation of an agar slope of his organism.

The term agar slope as a quantitative expression for a dose of bacilli is rather a vague one. From some estimations of the number of organisms present on an agar slope of average growth intensity, I adopted the number 10,000,000,000 as roughly expressing the number of organisms present.

A number of Roux flasks were inoculated with Strong's culture and the growth swept off in salt solution. The number of organisms present in the emulsion was determined by the direct counting method described in this *Journal* (Vol. XII. 1912, p. 362).

Twenty rats and twenty guinea-pigs were then inoculated subcutaneously with such a quantity of the emulsion as contained 10,000 million organisms.

A week later all the animals received a test dose of three million living virulent plague organisms from a passed culture that killed 90 % of normal rats inoculated with this dose.

Of the 20 guinea-pigs 13 died of plague and of the 20 rats 9 died of plague, a survival percentage of 35 in the case of the guinea-pigs and 52 in the case of the rats.

It has been previously shown in these reports that successfully to vaccinate against a body-strain the antigen employed must be prepared

from organisms grown as far as possible under conditions obtaining in the body. (This *Journal*, Plague Supplement III, Jan. 1914, p. 408.) The experiment was accordingly made of growing Strong's avirulent culture in a serum medium and again testing its immunising power. A culture was prepared in 10% serum broth, which was then flooded on to the surface of agar. Twenty-seven rats were vaccinated with 200 million of the resulting living organisms. A week later their immunity was tested with the standard dose of virulent plague. Eight rats died of plague as the result of the test, the remaining 19 surviving, a survival percentage of 73. This is a higher survival rate than that obtained from the previous series, notwithstanding that in the previous series the dose of organisms was 50 times as great.

It is concluded that the addition of serum proteins to the culture medium had materially enhanced the protective power of the culture, a result that is in complete accord with my previous experience.

Further experiments on this point are in progress.

The nucleoprotein obtained from Strong's culture.

From the same strain of organisms as that used in preparing the vaccine for the last experiment (serum broth strain) a series of Roux flasks containing nutrient agar flooded with heated horse serum was inoculated. The growth obtained was used in the preparation of a nucleoprotein extract (*v. supra*).

The toxicity of this extract was, for white rats, but slightly lower than that obtained from the standard virulent strain when grown under the same conditions.

Strong strain nucleoprotein		Virulent strain nucleoprotein	
Dose	Rat	Dose	Rat
0.5 mg.	1 died 2 days	0.5 mg.	1 died 1 day
	2 " 3 "		2 " 1 "
0.25 mg.	3 " 2 "	0.25 mg.	3 " 1 "
	4 " 3 "		4 " 1 "
		0.1 mg.	5 " 1 "
			6 " 2 days

The immunising power of this extract was also determined, 30 rats receiving 0.02 mg. as a vaccinating dose. Five days later all were tested with the standard virulent culture, three million organisms being given as in the other series; 18 rats died of plague, 12 or 40% only surviving.

This result is of interest as it indicated the amount of loss in immunising value that the nucleoprotein suffers during the process of

extraction. Roughly 0.02 mg. of nucleoprotein is contained in 200 million organisms. The two series of rats therefore received the same dose of nucleoprotein. (It will be remembered that the fact that the immunising power of the plague bacillus resided in its contained nucleoprotein was established in the first of these reports, this *Journal*, Vol. IX, No. 3, Nov. 1910.) A comparison of the survival percentages in the two series of rats 73%—40% affords an index of the deterioration of the antigen due to the process of extraction.

On the other hand the rats that received the whole organism, one and all suffered from abscesses at the site of inoculation, whereas the local reaction in the case of the rats that received the nucleoprotein extract was absent. This series of rats would easily have absorbed twice the dose of the extract, in which case it is reasonable to suppose the resulting immunity would have been correspondingly higher.

Strong found (*loc. cit.*) that his culture would immunise guinea-pigs. As we have seen, the survival rate when a dose of ten thousand million organisms (one agar slope) was given, was 35%. The survival rate found by Strong was considerably higher than this, averaging 75%. This suggests that the test culture used by him was not as virulent as the test culture employed by me.

Some further experiments were made with the living organism and with the nucleoprotein extract obtained from a growth in serum containing media.

Twenty-seven guinea-pigs received 200 million living organisms each as a vaccinating dose. The organisms were grown on agar. When tested in the same manner as before 24 died of the disease. In another series 29 guinea-pigs received 0.02 mg. of the nucleoprotein extract, of this series 27 died of plague when submitted to the standard test.

From these experiments I conclude that:

1. The immunity to the standard virulent culture obtained as a sequel to vaccination with living avirulent organisms grown on agar is less than that obtained after vaccination with the antigen prepared from the same virulent culture.
2. The immunity conferred on guinea-pigs is small but distinct. This is the only means available for producing any immunity in guinea-pigs.

LXXVI. THE INFLUENCE OF RACE ON THE EFFICIENCY OF THE ANTIGEN.

BY SYDNEY ROWLAND, M.A., M.R.C.S.,

Of the Lister Institute, London.

A FURTHER series of rats immunised with 200 million living avirulent organisms (Strong) presents some interesting comparisons with the series already mentioned. (See this *Journal*, p. 756.)

This series was tested not with the standard virulent body-strain but with a culture received from Dr Castellani, who isolated it from a case of plague during the recent small epidemic in Ceylon (*British Medical Journal* (1914), No. 2779, p. 752). This culture was not very virulent, killing only 50% of the controls.

Of 42 rats immunised with the avirulent culture 25 or 59% survived the test inoculation.

The controls showed a survival rate of 50% so that the degree of immunity conferred appears to have been trifling, and the result suggests that the degree of immunity produced by the 'Strong' antigen against the laboratory strain is greater than against the Ceylon strain. The laboratory strain was more virulent than the Ceylon strain (as shown by tests) so that I must conclude that the two strains differ as regards their antigens. If this is true it presents another complication in preventive inoculation against plague, for satisfactory results might only follow vaccination with the particular strain concerned in any particular epidemic.

It is possible that this phenomenon may account for the poor results obtained by the Dutch authorities with Haffkine's prophylactic in Java. (*Mededeelingen van den Burgerlijken Geneeskundigen Dienst in Nederlandsch-Indië* (1912), pp. 140—148.)

To further elucidate this point two series of rats were vaccinated. Series I with the antigen (nucleoprotein extract) obtained from the Ceylon strain, Series II with the antigen obtained from the laboratory virulent strain.

Of 49 rats vaccinated with the 'Ceylon' antigen 36% survived the test by the virulent laboratory strain, whilst of 47 rats vaccinated with the virulent laboratory strain antigen and tested with the virulent laboratory strain no less than 60% survived.

It would appear then that the antigens derived from the Ceylon strain are not entirely identical with those derived from the laboratory virulent strain and that cross immunisation is not complete.

LXXVII. ON THE FAILURE TO VACCINATE AGAINST A VIRULENT BODY-STRAIN EVEN WITH AN ANTIGEN PREPARED AS FAR AS POSSIBLE UNDER BODY CONDITIONS.

By SYDNEY ROWLAND, M.A., M.R.C.S.,

Of the Lister Institute, London.

As experience with vaccinating experimental animals (rats) in the laboratory has accumulated so the difficulties attending the work increase. One of the great difficulties I have experienced recently is the sudden and unexpected rise in virulence of the test culture. This culture has now been kept going in the bodies of living rats for nearly a year. Originally derived from a virulent agar strain it has been passed and repassed continuously. On the death of a rat the spleen organisms are isolated on agar, the surface of which is smeared with fresh heart blood. From the culture thus obtained, which is almost invariably a pure growth, a serum broth culture is made. Three million organisms from this growth have now for some time constituted the standard test for immunity. Such a dose kills 90% of unprotected rats in from three to four days. Recently, however, the virulence of this culture has risen to a still higher degree and at present it will kill ten rats out of ten in two days. The virulence of this culture is apparent at the post-mortem examination of the animals.

The extent to which the infection pervades the animal is astonishing. The local reaction extends up the entire side of the rat, the pelvic glands and spleen even after this short period are a closely packed mass of bacilli. After a somewhat extensive acquaintance with the appearance of rats dead of plague, in the course of which I have examined many thousands of animals, I have never seen, even during the height of a Bombay epidemic, animals so severely infected. Advantage was taken of this rise in virulence of the test culture to determine the immunity conferred against it by an antigen prepared from it.

For this purpose two antigens were prepared.

Antigen No. I was prepared from a growth of the organisms on nutrient agar flooded with fresh rat serum, antigen No. II was prepared in the same manner with the exception that an intermediate culture in fresh rat serum was interposed.

With the first antigen 44 rats were inoculated. The dose was 0.02 mg. Of these 44 rats 38 succumbed to infection when tested five days later with three million organisms.

With the second antigen 49 rats were inoculated, using the same 0.02 mg. dose. Of these 34 died of plague when tested five days after the inoculation of the antigen.

Now these results are in striking contrast to those detailed in a previous Report (see this *Journal*, p. 753), where it was shown that a considerable immunity followed the inoculation of the same dose of an antigen prepared from the same stock of organisms after three passages through rats, when tested against a strain of corresponding virulence.

In practice strains of organisms of every possible degree of virulence will assuredly be met with, and should a strain corresponding to the one with which the above experiments were made be prevalent in a local epidemic, complete failure of any protective inoculation at present known will occur.

LXXVIII. THE PROTECTIVE AND CURATIVE VALUE,
AGAINST INFECTION WITH A SERUM RACE OF
PLAGUE, OF THE SERUM OF A HORSE IMMUNISED
WITH NUCLEOPROTEIN EXTRACTED FROM A STRAIN
OF PLAGUE BACILLI PROPAGATED ON SERUM
PROTEIN.

By SYDNEY ROWLAND, M.A., M.R.C.S.,
Of the Lister Institute, London.

I HAVE shown in a previous report (this *Journal*, Vol. XI. Dec. 1911, pp. 11 *et seq.*) that a protective and curative serum for rats can be prepared against a broth (laboratory) strain of plague, by means of immunising horses against a toxic nucleoprotein derived from the bodies of the same strain of plague bacilli, also propagated upon broth. The administration of such a serum in doses of 1·0 c.c. subcutaneously, completely protected a series of rats, which had received, subsequent to inoculation, a standard test lethal dose of broth organisms. The serum, moreover, exercised a curative action in the case of another series of rats, when administered in 0·5 c.c. doses, 6—30 hours after inoculation with the usual test dose, reducing the mortality from 80 to 18 %. When, however, immune serum prepared in the above manner was tried in practice in Bombay (this *Journal*, *ibid.*, pp. 326 *et seq.*) in cases of human pest, the results obtained were very discouraging and failed to show any effect whatever in influencing the course of the disease.

It was suggested to me by Dr. Martin, of the Lister Institute, that the explanation of this seeming paradox lay in the fact that the serum that gave such good results on rats in the laboratory failed in Bombay because the strain of organisms with which the serum was prepared and against which it was tested was a laboratory or broth strain. Acting on this suggestion it became of interest to ascertain whether a serum could be prepared using as a source of the immunising toxin a body-strain of organisms and using the same strain as a test of the efficiency of the serum.

The horse "Wealdstone," that was used for the preparation of the first batch of serum, was accordingly taken in hand.

A bleeding before the immunisation was commenced was tested both against a body-strain and against a broth strain of plague with the results shown in the table :

<i>Broth race 3,000,000 organisms</i>			
Dose serum 24 hours before test		Dose serum 24 hours after test	
1 c.c.		1 c.c.	
10 rats, 3 died pest		10 rats, 5 died pest	
0.1 c.c.	[Controls 10 rats,	0.1 c.c.	
10 rats, 7 died pest	8 died pest]	10 rats, 4 died pest	
<i>Serum race 3,000,000 organisms</i>			
1 c.c.		1 c.c.	
10 rats, 5 died pest		10 rats, 10 died pest	
0.1 c.c.	[Controls 10 rats,	0.1 c.c.	
10 rats, 10 died pest	10 died pest]	10 rats, 10 died pest	

This experiment shows that, as had been suggested, the serum of the horse Wealdstone (which was the serum that was sent to Bombay for Clinical experience) was still operative against a broth strain of plague but absolutely without effect against a body-strain of plague. If the test above given be compared with that given in the previous report (this *Journal*, *loc. cit.*) it will be seen that the serum was of the same order of potency as on the occasion of the last test.

These results also emphasise the difference between a serum race and a broth race, for whereas 0.1 c.c. of the serum was followed by the survival of six out of ten rats when given twenty-four hours after the test dose of broth organisms, the same quantity of serum was without effect against the same number of serum organisms (body-strain) which had been inoculated into ten rats the same time before the inoculation of the serum.

The immunisation of the horse was then proceeded with.

The material used for the immunisation was a toxic nucleoprotein extract prepared from a body-strain of plague grown in horse serum on the surface of agar in Roux flasks. (For method *vide* this *Journal*, Vol. x. Nov. 1910, pp. 536 *et seq.*)

The horse received, intravenously, doses of this extract as follows :

16—2—14	10 milligrammes
18—2—14	30 „
23—2—14	10 „
27—2—14	50 „
4—3—14	200 „
10—3—14	400 „
17—3—14	800 „
28—3—14	Bled 1 litre.

The immediate effect on the horse after each dose was, especially after the larger doses, prostrating. This soon passed off and as a rule the horse was in his usual form in the course of two days.

The test of the bleeding taken on 28—3—14 is given in the table. This test was against the same body-strain as that used for the previous test. During the time the immunisation of the horse lasted, the strain was passed continuously through rats, being isolated at intervals on fresh rat blood agar.

The number of plague organisms used for all these tests was three millions.

Test of bleeding 28—3—14 against <i>serum race</i> , 3,000,000 organisms		
Dose serum 24 hours before		Dose serum 24 hours after
1 c.c.		1 c.c.
10 rats, 4 died pest		10 rats, 3 died pest
0.1 c.c.	[Controls 10 rats, 10 died pest]	0.1 c.c.
10 rats, 6 died pest		10 rats, 5 died pest
0.01 c.c.		0.01 c.c.
10 rats, 8 died pest		10 rats, 10 died pest

The effect of the immunisation of the horse has been to render its serum operative against a serum (body) strain of plague, against which, before the immunisation, it was inoperative.

For whereas before the immunisation, of ten rats that received 1 c.c. 24 hours after the administration of the test dose, all died of plague, after the immunisation, of ten rats that received the same dose the same time after the test inoculation, only three died of plague.

Similarly with the smaller doses of serum. After the immunisation, 0.1 c.c. of serum saved five rats out of ten that had 24 hours previously received the test dose of the body-strain. Before the immunisation this dose of serum was absolutely without effect under the same conditions, not only so but ten times this dose, 1.0 c.c., was similarly inoperative.

It is concluded that just as with a broth race it is possible to prepare a curative serum against a broth race, so with the body-strain it is possible to prepare a serum operative against a body-strain.

NOTE.—It is of interest to note that the amount of toxin that was administered to the horse intravenously on 17—3—4, viz. 800 milligrammes, was contained in the growth from about 300 Roux bottles, an amount that is quite impossible to administer if given wrapped up in the bodies of the bacilli. This is one of the advantages of using the nucleoprotein extract. Given in the form of an emulsion of bacilli this amount of toxin would be contained in about three litres of thick emulsion. To put three litres of thick emulsion into the vein of a horse would probably prove immediately fatal.

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LXXIX. ULTRAVIOLET LIGHT AS A GERMICIDE IN THE PREPARATION OF PLAGUE VACCINE.

BY SYDNEY ROWLAND, M.A., M.R.C.S.,

Of the Lister Institute, London.

IN the course of these reports it has been shown that: (1) An efficient protection against a laboratory strain of plague is easily obtained by vaccination with a nucleoprotein extract obtained by the dehydration method (this *Journal*, Vol. x. No. 3, Nov. 1910, pp. 536 *et seq.*). (2) An immunity of a high order may be obtained against a body-strain of plague by the use of a nucleoprotein extract, provided the organisms furnishing the extract are of the same strain as those against which it is desired to vaccinate (this *Journal*, Plague Supplement III, Jan. 1914, pp. 403 *et seq.*).

The use of heat being inadvisable in the preparation of such vaccines owing to its deleterious effects on the antigen, and the preparation of the nucleoprotein extract requiring a somewhat special equipment for its successful employment, other means were sought whereby it might be possible to destroy the infectivity of a vaccine without at the same time destroying the contained antigen. Could such means be found the preparation of a vaccine might be greatly simplified. The use of a whole vaccine entails in practice certain well-known conveniences of manipulation. The desideratum being then some method of killing a culture of enhanced virulence (body-strain) without at the same time so deteriorating the antigen contained within it that it is rendered useless, the well-known bactericidal action of ultraviolet light suggested itself as a method worth investigating.

Experiment 1 (Orientating experiment).

A thick emulsion of virulent bacilli obtained from the spleen of a rat dead of plague, isolated on blood agar, was prepared in salt solution. (A similar emulsion was used throughout these experiments.)

This was exposed to the light of a 5 ampere arc for one hour, using a quartz cell and a quartz collecting lens.

Ten control rats inoculated with 20 million organisms. All died of plague.

Ten control rats inoculated with the same dose that had been exposed to the same radiation employing a glass collecting lens, all died of plague.

Of ten rats inoculated with the same dose of the emulsion exposed to the radiation collected by a quartz lens, one died of plague.

This result shows that the bactericidal radiation is stopped by glass, *i.e.* that the active rays are of shorter wave length than about 330 micro mu.

Experiment 2.

The same experiment was repeated, using a quartz collector, and the time of exposure was varied from 40 minutes to five minutes. Of the entire series of rats inoculated with a dose of 20 million organisms all survived.

As short a time as five minutes is therefore sufficient to kill the plague bacillus when exposed to the radiation from a carbon arc.

Experiment 3.

The same experiment was repeated, exposure times as short as one minute being employed.

The results are shown in the following table. Six rats were in each case inoculated with 30 million organisms.

Controls	all died of plague	
1 min. exp.	4	" "
2 "	5	" "
3 "	4	" "
4 "	1	" "
5 "	1	" "
8 "	0	" "
10 "	0	" "

The previous result was thus confirmed.

The survivors were then, after an interval of ten days, tested for immunity. All died of plague.

The inoculation of 30 million organisms, killed by ultraviolet light, had therefore produced no immunity.

Experiment 4.

The last experiment was repeated, using nickel cored carbons which afford a particularly rich ultraviolet spectrum, with the following results:

Controls	all died of plague		
1 minute	5	„	„
2 minutes	3	„	„
4 „	0	„	„
10 „	0	„	„

The survivors were then, after an interval of ten days, tested for immunity. None was evident.

There were two survivors from the six rats that had received the organisms submitted to two minutes' exposure, and two from the six rats that had been submitted to four minutes' exposure to the radiation.

Experiment 5.

The last two experiments were repeated, using the radiation from a quartz mercury lamp.

No. rats	Min. exposure	Fate after test
6	1	2 survived
6	2	1 „
6	4	2 „
6	10	0 „

All the rats survived the preliminary inoculation with the radiated organisms.

The conclusions to be drawn from these experiments are that: (1) Ultraviolet light kills plague bacilli with facility. (2) The killing of the organisms is accompanied by destruction of the antigen. There was still a chance, however, that by using rays of definite wave length a differential effect might be obtained, and that a wave length could be found that whilst being bactericidal was not capable of injuring the antigen.

I therefore set about to ascertain the wave length of the rays responsible for the killing.

The spectrum of the light emitted by a quartz mercury lamp projected by a quartz prism and a simple lens of 40 cm. focal length was employed.

The spectrum was projected on to the surface of an agar plate previously inoculated with a virulent strain of plague. It was found

that after half an hour's exposure no growth took place beyond the line 296 micro mu.

This experiment was repeated several times and the point established that wave lengths shorter than this were uniformly bactericidal up to the extreme limit of the spectrum that the apparatus would yield, i.e. up to about the line 237 micro mu. We may say then that the bactericidal region of the available spectrum extends between the lines 237 and 296.

The method of plating was controlled by a direct observational method, using a broth culture of *Bacillus enteritidis*. The spectrum was projected into the quartz substage condenser of the microscope and the preparation illuminated by a subsidiary lamp, the light from which was reflected from the last face of the quartz prism. Matters were so arranged that any desired wave length could be employed. A particular organism was selected and its movement followed with the eye. By opening a shutter controlling the ultraviolet light at the desired moment the effect of any wave length on the motility of the bacillus could be observed.

Working in this way it was found that motility was immediately arrested as soon as the region 2967 was reached and that the effect was still recognizable as far as the region 2557, after which it became so slight as to be uncertain.

This experiment, which was repeated very many times, always led to the same result.

Further observations were made on infusoria and on algae and desmids. The effect of the bactericidal rays on these organisms was often very startling. One particular infusorian employed (*sp. incert*) which contained a large number of chloroplasts, on being submitted to the effect of the rays immediately burst, scattering the chloroplasts. A diatom will under the same circumstances separate its valves and extrude its contents, an alga will contract its chlorophyll bands into a central tangled mass and many other unicellular organisms will behave in curious and unexpected ways.

So far, then, the possibility of preparing a vaccine by the help of these extraordinarily energetic rays did not appear hopeful. The violence of their action was unexpected, but it is well known that many ferments are destroyed by ultraviolet light.

The next point to be determined was the extent of the spectrum that was absorbed by the antigen, which for this purpose may be taken as the nucleoprotein extract prepared by the sulphate method. The

absorption spectrum of this substance was accordingly determined, and it was found that absorption commenced at the line 2967 and extended to the extreme ultraviolet.

Now the line 2967 is the last line that permits the bacillus to grow when exposed to the influence of the complete spectrum as we have above pointed out. It appears then that that region of the spectrum which is responsible for the bactericidal effect is precisely that region that is absorbed by the antigen. It was still barely possible that there existed wave lengths that while being absorbed by the antigen were bactericidal but not capable of injuring the antigen. This was a bare possibility, but no more.

The experiment was accordingly made of exposing an emulsion of bacilli to the entire radiation of a quartz lamp through the (query) protective action of a solution of the antigen. Thirty rats were inoculated with a vaccine prepared in this manner, the exposure varying from five to 20 minutes. Subsequent test with a virulent culture, which killed all out of ten controls, also killed the whole of the 30 inoculated rats.

It is concluded from these experiments that the bactericidal and antigen injuring regions of the spectrum are identical¹.

I wish to express my profound sense of obligation to Dr. C. J. Martin, Director of the Lister Institute, for his help throughout the entire work on the results of which these reports are based; also to my colleagues on the staff of the Lister Institute, most of whom have helped me in one way or another during the progress of the investigation.

¹ At this point Dr. Rowland's investigations were interrupted by the European War.

No. 21

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STUDIES ON THE EFFECT EXERTED BY SHAKING ON SERUM.

By HANS SCHMIDT

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INTRODUCTION.

OWING to the difficulty of satisfactorily explaining many facts in serological work, there has been a tendency in the last few years to consider serological phenomena more and more from a physico-chemical point of view.

A serum, physically considered, is a protein hydrosol containing electrolytes. Different proteins, globulins and albumins, are kept in the serum in the relatively stable state of an hydrophile colloidal solution both by their mutual protecting influence as well as by the influence of the salts. Under the most diverse conditions of a purely physical nature, such as heat, storage, freezing, decrease of salt concentration by dilution, or loss of salts by dialysis, increase of the surface by shaking, etc., the stability of the colloidal phases is altered and more or less energetic alterations of the physical state of the serum take place. These may be reversible or irreversible and are followed by changes in the serum as regards their serological properties.

Shaking a serum produces well-marked alterations in it, visible to the naked eye first of all as cloudiness and followed later by precipitation. The production of cloudiness in a serum by shaking has been already observed by Jakoby and Schuetze (1910), but not until recently in the work of P. Schmidt and M. Liebers (1913), H. Schmidt (1913) and L. Hirschfeld and R. Klinger (1914) has this phenomenon been especially taken into account and recognised as the main alteration which a serum undergoes during shaking and of which all other changes observed are more or less a consequence. Seeing the increase of surface to be the main effect of shaking, and that the mechanical agitation by itself plays

a less important part, probably only in continuously renewing the surface, it may be assumed that the alteration in the serum as the effect of shaking occurs more quickly the greater, *ceteris paribus*, the surface is, to which the serum is exposed. It is therefore evident that the intensity of the shaking influences greatly the time necessary to produce cloudiness in a serum, for the more intensely shaken a serum is, the finer is the froth and the greater is the development of the surface. S. and S. Schmidt-Nielsen (1909), in their experimental work on the inactivation of rennet by shaking, have observed to what extent the difference in the ratio of shaken liquid to bottle volume produces a variation of the results. Ritz (1912) also drew attention to this relation as regards shaking of serum and in a former paper (1913) I fully confirmed this.

In order to obtain comparable results, the ratio of bottle volume and liquid as well as the intensity of the shaking movement must be kept constant.

Factors concerned in the effect of shaking upon serum.

Supposing that these relations are kept throughout under optimal conditions, there are still many other factors which may by their presence either accelerate or inhibit the effect of shaking on serum. Before quoting them it should be mentioned that all the following observations are made on fresh guinea-pig serum. The complementing power of such a serum is a characteristic property of its freshness and will become lost by any factor which may alter the physical conditions of the serum proteins. The influence which shaking especially exerts upon the complementing power of a serum will be dealt with in a further communication. It is proposed here merely to deal with the effect of shaking in producing coagulation and denaturation of the proteins of a serum.

The concentration of the serum.

In the first experiments made to inactivate the complement of a serum by shaking, it was found by Jakoby and Schuetze (1910) that a longer and more intense shaking is required to render a serum inactive when undiluted, and therefore these authors used the usual dilution of 1 : 10 in their experiments. This dilution has been later found by Ritz (1912) to be an optimum, and my results, published in a former paper (1913), partially confirmed this. In the meantime I found, however, that frequently a serum diluted 1 : 20 or even 1 : 40 with 0.85 % saline

showed the same degree of cloudiness and inactivity as regards complement action as when diluted 1 : 10. If the inactivation by shaking is due to change in surface energy, I can see no reason for an optimal dilution. The process is to be considered as progressing with time. The shaking causes aggregation and coagulation of a certain part of the proteins, as will be more fully explained later. This coagulation affects only a part of the total proteins. Now, since the relation between volume of liquid and gas as well as the shaking movement are kept constant, thus producing by the equal intensity of shaking an approximately uniform froth and therefore an approximately similar extent of surface, the relation of the denaturated protein to the total protein-content will be the smallest in the undiluted serum, which therefore needs a greater time to become altered to the same extent as in the diluted serum. The relation of the amount of the unchanged proteins to the denaturated proteins increases with the concentration of the serum, as Schmidt-Nielsen also found in the inactivation of rennet by shaking. If the complementing power of the serum be taken as a measurement of the effect produced by shaking, the decrease of complementing power by shaking will be partially covered by that produced by dilution. In the case of a serum the conditions are more complicated than in the case of ferments as rennet or pepsin, etc., inasmuch as the shaking produces principally a denaturation of the euglobulins, as well as an alteration of the albumins, which latter play an important part in the stability of the globulins. Since dilution of the serum decreases the absolute content of albumins, this factor must be taken into account, when considering the more rapid inactivation of diluted sera effected by shaking.

Concentration of salts in the serum.

If instead of 0.85 % saline, dist. water is taken as dilution medium, or if NaCl crystals are added to the native serum, the concentration of salts will be altered and, apart from the effect of shaking, this alone will account for a change in the stability of the colloidal proteins.

a. Decrease of salt concentration.

Sachs and Teruuchi (1907) found that dilution 1 : 10 with aqu. dist. and standing $1\frac{1}{4}$ hours at 37° C. will affect a complement-containing serum in such a manner as to render it inactive, if it is again made isotonic. They found that this phenomenon took place more rapidly

at higher temperatures and that a dilution of 1 : 10 was an optimum. Such a serum could be reactivated by addition of the albumin fraction obtained by dilution and acidification with CO_2 , while the euglobulin fraction was rarely able to do so, and only then to a lesser degree [Sachs and Bolkowska, 1910]. Marks (1912) found that a serum after dilution to 1 : 10 with aqu. dist. and standing $1\frac{1}{4}$ hours at 37°C . was not completely inactive. The fractions of a serum so treated obtained by CO_2 gave full action, when combined with the corresponding fractions of a normal serum. Bessemans (1913) extended these observations and found that the fractions were inactive when combined with themselves. If the fractions of a normal serum were diluted 1 : 10 with dist. water and then kept for $1\frac{1}{4}$ hours at 37°C ., he found, generally speaking, no loss of power in their action. Sometimes a slight reduction could be observed in the action of the globulin fraction, as also observed by Guggenheimer (1911), but no alteration of the albumin fraction ever occurred. Diluted with aqu. dist. produces decrease of the salt concentration. This alters the conditions controlling the solubility of the euglobulin, part of which is therefore thrown out of solution in the form of a suspension, which renders the serum opalescent and cloudy. Addition of salt brings the suspended euglobulin promptly back in solution. After standing a long time however the suspended and precipitated euglobulins undergo alterations, becoming insoluble. The solubility of the euglobulins is favoured by the presence of serum albumins. A solution of euglobulin obtained only by means of salt is always more or less opalescent, indicating that there is no true solution. According to H. Chick (1913) electrically neutral compounds of the salts with the euglobulin are formed, which Schryver (1910) supposes to be due to adsorption. In the serum the albumins, owing to their lower surface tension value, exert a protecting influence on the euglobulin in solution, causing a greater dispersity and thus a true solution of the latter. A more detailed reference to these relations will be given later.

In view of the importance of the relationship between the effect of dilution and standing and the effect produced by shaking, I repeated the experiments of Sachs and Terunchi and give below the results obtained.

Technique :

Haemolysis was due to the complementing action of the serum in combination with sensitized sheep red corpuscles, of which a 2.5 % emulsion in 0.85 % saline was used. Sensitization was effected with three times the single lysing dose of an inactivated rabbit immune serum.

The following schema has been adopted to illustrate different degrees of haemolysis :

-  No haemolysis.
-  Faint trace of haemolysis.
-  Very slight haemolysis.
-  Well-marked haemolysis.
-  Half haemolysed.
-  Strong haemolysis.
-  Very strong haemolysis.
-  Nearly complete haemolysis [a slight trace of unlaked cells at the bottom].
-  Complete haemolysis.

Experiment showing the influence of dilution with dist. water upon a complement-containing guinea-pig serum.

Fresh guinea-pig serum was diluted 1 : 10 with aqu. dist. and kept for $1\frac{1}{2}$ hours at room temperature and at 37° C. Then the serum, rendered isotonic, was tested for its haemolytic power with 0.5 c.c. sensit. red cell emulsion (AB dose = 3 times the single lysing dose).

Haemolysis after 1 hour at 37° and 15 hours at room temperature.
Total volume in each tube 2.0 c.c.

		1.0	0.5	0.25	0.15	0.0
C_n = normal serum dil. $\frac{1}{10}$ with NaCl		...				
C_r Dil. $\frac{1}{10}$ with aqu. dist. $1\frac{1}{2}$ hours at room temp. then rendered isotonic	C_r ...	...				
	+ 0.5 c.c. E_n					
	+ 0.5 c.c. M_n					
C_t Dil. $\frac{1}{10}$ with aqu. dist. $1\frac{1}{2}$ hours at 37° , then rendered isotonic	C_t ...	...				
	+ 0.5 c.c. E_n					
	+ 0.5 c.c. M_n					

The thus treated sera, before being rendered isotonic, were treated by CO_2 according to Liefmann's method. The globulin fraction, = M-piece, was not taken up in solution by 0.85 % NaCl until immediately before use. The albumin fraction, = E-piece, was filtered through hardened paper and then rendered isotonic.

The fractions thus obtained from C_n , C_r , C_t are respectively E_n , E_r ,

E_t , M_n , M_r , M_t . 1 c.c. of each gave with 1 c.c. of each other and 0.5 c.c. of the sensit. red cell emulsion, the following reactions :

E_n + saline	●		E_n + M_t	■
M_n + ..	□		E_n + M_r	●
E_n + M_n	■		E_r + M_n	■
E_r + saline	●		E_r + M_t	□
M_r + ..	□		E_t + M_n	■
E_r + M_r	●		E_t + M_r	□
E_t + saline	□			
M_t + ..	□			
E_t + M_t	□			

The experiment shows that inactivation has taken place, but more completely in the case of the serum which has stood at 37° C. But contrary to the statement of Teruuchi, I found that a fresh globulin fraction of a normal serum exerted a better restitution effect than the albumin fraction.

As regards the CO_2 -fraction of the serum, treated by dilution with aqu. dist. and standing, it follows from the above experiment that the alteration concerns only the globulin fraction, the albumin fraction of a serum thus treated proving to be active when combined with a normal globulin fraction.

If the serum however was not fresh, I found that dilution with dist. water and standing had a far less destructive effect upon the complementing power of a serum. Inactivation will not be complete, even after many hours standing. Cf. Exp. 6 in my paper on the effect of Berkefeld filtration upon Complement (this *Journal*, 1914). What process has taken place during dilution and standing so as to render the complementing power of a serum inactive is difficult to say, for the suspended globulins seem to be in no way altered, the serum becoming quite clear if rendered isotonic. Probably some alteration in the H⁺-concentration takes place, which may inhibit complementing action, but no definite evidence can yet be given in favour of this conception.

Now, if such a serum, the euglobulins of which are in a state of lability owing to lack of sufficient salt concentration, be shaken, it is evident that these euglobulins will aggregate and coagulate more readily.

In fact, a serum diluted 1 : 10 with aqu. dist. is very rapidly altered by shaking, so that the cloudiness, which in the unshaken control serum will promptly vanish by addition of salt, will not disappear if the serum be rendered isotonic, but will do so on the addition of alkali, thus indicating that the protein particles have undergone denaturation.

Increase of salt concentration.

An increase in the concentration of salt has a somewhat opposite effect upon the influence of shaking. Since Friedberger (1908) drew attention to the use of concentrated salt solution for the preservation of the complementing power of a serum, this method has been frequently and successfully used [Hecht, 1910]. As already mentioned, a certain concentration of salt is necessary for the stability of the euglobulin in the serum [H. Chick (1913)]. This protection however is only a relative one, as the euglobulin is also precipitated in the course of time in a native and otherwise untreated serum. Higher salt concentration prolongs the time during which a serum can be kept clear, by maintaining the dispersity of the protein-phases and thus preserving the complementing power.

If a serum is shaken in a hypertonic condition the time necessary to cause a denaturation and coagulation of proteins will be found to be prolonged, but even as high a concentration as 20 % will not absolutely protect the serum, as well as the isolated protein solutions, from becoming denaturated, as will be seen later. The protecting influence of salts upon the effect of shaking is therefore relative, which fact has already been recognised by Hirschfeld and Klinger (1914) to be the case in human serum.

The influence of temperature upon the effect of shaking.

The accelerating influence of a temperature of 37° C. upon the effect of shaking has been observed by all authors who have worked on the shaking inactivation of complement and ferments. The denaturation and precipitation of a part of the serum proteins occur also at low temperatures. Seeing that it is principally a surface phenomenon, this would be expected to be the case. The acceleration observed at 37° C. is a summation of the effects produced by the temperature and that produced by the shaking. An explanation of this very complicated process will be attempted later. Here, only the fact may be mentioned.

If, however, on the other hand, a serum has been previously heated, the time required to produce cloudiness and coagulation by shaking will be found to be prolonged, the more so the greater the temperature to which the serum was heated. This observation has only been made by Hirschfeld and Klinger (1914) in the case of human serum. They found that exposure to a temperature of 58°C . for 1 hour prevented the serum from becoming cloudy on shaking, as well as by dilution with aqu. dist. They postulate that thermoinactivation as well as a hypertonic condition, the latter in a lesser degree, renders the serum euglobulins more stable; an explanation of this fact however was not given. I think a probable explanation can be given by the results of observations made by H. Chick and C. J. Martin (1913) and H. Chick (1913) in the following way.

The denaturation process of proteins as the result of high temperatures has an abnormally high coefficient of temperature, which apparently effects the denaturation of proteins and their coagulation in the serum to be due to a certain temperature. In reality the process of denaturation takes place already at lower temperatures, but proceeds very slowly. It can however be observed to take place at 37°C . under favourable conditions. The euglobulins form salt compounds in the serum, which remain electrically neutral, when enough salt is present to prevent their dissociation. If the euglobulin has been denaturated by heat, and there is evidence that an exposure to 56°C . for half an hour is sufficient to produce heat-denaturation, the euglobulins lose their characteristic property of forming electrically neutral solutions with salts. The euglobulin particles become electrically charged and any change in dispersity is prevented by their mutual repulsion. The alkalinity of the serum is increased. A decrease of H^+ -concentration takes place [S. T. Sørensen and Jürgensen (1911)], and the dispersed protein particles are negatively charged. As long as in such a serum the H^+ -concentration is kept constant, the stability of the euglobulins remains increased.

This may probably be the principal reason why sera previously heated remain clear much longer on standing and require a considerably longer time to become visibly affected by shaking.

I have often observed in the case of guinea-pig serum that half an hour's heating at 56°C . does not prolong very much the time necessary for coagulation by shaking, but a longer exposure to 56°C . prolongs considerably this time, and I was unable to bring about a visible coagulation by shaking in the opalescent serum diluted 1:10 with saline and exposed to the temperature of boiling water. It may be possible that

the resistance against the shaking effect, which can be observed in heated sera, may be partly due to the influence of capillary-active substances, which are formed in the serum during heating and which lower the surface tension against air. Further reference to this will be made later.

Influence of the nature of the gas.

That the coagulation of proteins by shaking is not dependent on the nature of the gas, which is in contact with the serum surface, in so far as it is chemically inactive, has already been shown in the early experiments of Melsens (1851), Harting (1851) and Smee (1863, 1864). Metcalf (1905) stated that the formation of membranes of the surface of colloidal solutions was independent of the oxygen and Ramsden (1894) found that all solutions, the particles of which coagulate under the influence of shaking, show this phenomenon also, if oxygen is excluded. Similar results were obtained by A. O. Shaklee and Meltzer (1909), who found that the inactivation of pepsin by shaking took place equally well whether the experiment was done in O, H, or in CO₂. Contrary to this, Courmont and Dufourt (1912) concluded from their experiments that the inactivation of the complement in a guinea-pig serum by shaking was due to oxidation. This could not be confirmed by P. Schmidt and M. Liebers (1913) nor by myself (1913) working independently. No evidence was found for oxidation being responsible for the shaking effect. However, instead of shaking the serum in oxygen, as I formerly did, in order to determine what rôle, if any, oxidation plays in shaking inactivation, I exposed the complement-containing serum to the influence of oxygen in nascent state obtained by the action of the serum katalase on hydrogen peroxide. I found that if H₂O₂ was added to the serum in such quantities as to produce an 0.5 % H₂O₂-solution, no evidence of loss of complement could be observed in spite of the large amount of oxygen thus developed in the serum, enough being present to render the serum colourless in a few minutes. I am therefore convinced that the effect of shaking on serum is not due to oxidation.

Influence of the age of the serum upon the shaking effect.

Storage of a serum diminishes its complementing power. This loss runs parallel with the formation of a cloudiness and of a precipitate. Apart from the influence of bacterial growth, which according to Hara (1913) accelerates the loss of complementing power, this decrease of complement and the formation of precipitates occur also under sterile

conditions. The precipitates in old sera consist of euglobulin, which has become more and more insoluble. It has been seen that hypertonic condition as well as previous heating increase the stability of the euglobulin to this extent, that sera so treated remain clear for a considerably greater length of time. If a serum which has been kept for a long time be treated by CO_2 according to Liefmann's method, I found that the action of the fractions, thus obtained, varied according to the length of time the serum has been kept. Great individual variations however occur. Generally speaking I found, at the commencement, when the complement activity was not yet completely lost, that both fractions gave a complete action when combined with the corresponding fractions of a fresh serum. Later on the globulin fraction of the old serum loses its power to reactivate a fresh albumin fraction, but not *vice versa*. Finally the albumin fraction of the stored serum loses its action. This however was not found to be of constant occurrence, for sometimes a serum was found, the globulin fraction of which retained its activity with a fresh albumin fraction as long as the albumin fraction did with a fresh globulin fraction. This however must be considered as exceptional. Changes taking place in the euglobulins in the course of storage have been already noted by Liefmann (1911) and Mutermilch (1911). They found that in sera kept for a long time the albumin fraction was the more stable of the two, and this has been confirmed by Bessemans (1913) more recently.

A probable explanation of the process is, that first of all an adsorption of the albumins in the free surface takes place. This size of the surface is of importance, for I could observe that with an increase of the surface against air there is an increase in the rapidity with which the complement of a serum is destroyed. This adsorption of albumins on the surface diminishes the stability of the euglobulins, leading to a gradual precipitation of the latter. At the same time adsorption of salts is possibly taking place, for according to Hecht (1914) the electrical conductivity decreases with the length of time.

The process of inactivation due to storage then closely resembles that produced by shaking. The same alterations occur, but more rapidly, and are therefore followed by a much more rapid loss in the inactivity of the albumin fraction.

It is interesting to notice that the greater stability of the euglobulins produced by heating is paralleled by the observation of Mutermilch (1911), who found that under similar conditions the time required to produce precipitation of the euglobulin by dialysis is considerably

prolonged. The same but to a lesser degree has been found by him (1911) to be the case in an old serum.

This is precisely what occurs with regard to the production of a precipitate in a serum by means of shaking, a well-marked inhibition occurring in the case of a heated serum and a relatively lesser inhibition in an old serum.

The effect of shaking upon the isolated proteins of a serum.

In regard to all these factors, which either accelerate or tend to inhibit the effect of shaking, it is highly probable, that the shaking of a serum results in rendering the euglobulins unstable and thus increasing their tendency to aggregate and precipitate. That the effect of shaking is really of such a nature is rendered certain by a study of the effect of shaking upon the isolated proteins. I undertook first of all experiments showing the effect of shaking upon the isolated fractions, obtained by Liefmann's method [dilution with dist. water and acidification with CO_2]. The precipitate which, re-dissolved in 0.85 % NaCl, represents the globulin fraction, contains the euglobulin and a relatively small part of the pseudoglobulin, which can be demonstrated by the behaviour of the solution, when 1 : 3 or 1 : 2 is saturated with the sulphate of ammonium.

The same method of salting out reveals the fact that the albumin fraction of the serum, *i.e.* the supernatant fluid rendered isotonic, contains all the albumin and the main bulk of the pseudoglobulin.

Now, if the albumin and globulin fractions are shaken under similar conditions the following results are obtained.

The shaking of the globulin fraction produces relatively little and unstable froth, and in a very short time cloudiness is developed and precipitation takes place. If the precipitate is removed by centrifugation, and the shaking continues, no more cloudiness will appear, the liquid remaining clear. Half saturation with the sulphate of ammonium reveals the presence of some pseudoglobulin, euglobulin no longer being present. If the salt concentration of the globulin fraction is increased to 20 %, the time required to coagulate the euglobulins is found to be prolonged but coagulation finally does take place.

The shaking of the albumin fraction however is accompanied by the production of a copious and persistent froth and the liquid is found to be still quite clear at a time when the euglobulins of the globulin fraction are to a large extent precipitated. Prolonged shaking however produces coagulation of some of the proteins in the albumin fraction, also the

smaller the quantity of salts present, the more rapidly does this occur. In any way, the amount of protein in the shaken albumin fraction found to be coagulated is very small compared with the big precipitate in the globulin fraction.

In order to decide, whether this coagulated protein of the albumin fraction is pseudoglobulin or albumin, these serum proteins were prepared and tested separately. I obtained these preparations by third, half and total saturation with the sulphate of ammonium, and the euglobulin as well by dilution of a serum and acidification with acetic acid up to its isoelectric point. The proteins thus obtained and freed from the ammonium sulphate by dialysis, were then shaken under varied conditions as to protein—or salt concentration. Summarising my results I can say :

The production of froth was well marked in the albumin solution, to a slightly lesser extent in the pseudoglobulin solution, but the euglobulin solution gave a froth only as long as shaking was going on and this froth being unstable quickly disappeared. This is interesting in regard to the different influence which these proteins exert upon the surface tension of water. Cloudiness followed by precipitation was first observed to occur in the euglobulin solution, then in the pseudoglobulin solution, and finally in the albumin solution, the latter requiring eight hours' shaking. Higher salt concentration tended to inhibit the precipitation, but this inhibition was less marked in the case of euglobulins.

In view of the great difficulty of getting absolute separation of the different proteins, I am far from considering the proteins, prepared as described, as being pure. I therefore repeated the experiments with preparations of albumin and globulins of horse serum, very pure preparations, which Dr Hartley kindly put at my disposal. I shall refer to these preparations later on in regard to the surface tension. The shaking effect of these proteins was similar to those described above, the albumins requiring the longest time to be precipitated. P. Schmidt (1914) did not evidently shake the albumin long enough, for he did not succeed in getting cloudiness and precipitation.

In spite of the certain restrictions which are to be observed in applying these observations to those observed when shaking the whole serum, I am inclined to consider it highly probable that the euglobulins in the serum are the first proteins which are denaturated and precipitated by shaking. Some alterations in the albumins must also have taken place, because their influence on the dispersity of the euglobulins must

have been lessened. If albumins either in the form of a CO_2 -albumin fraction, or in pure state, be added to the serum before shaking takes place, this excess of albumin prolongs the time necessary for the precipitation of the euglobulin, and *vice versa* an excess of globulin is found to shorten this time. Only when a serum is shaken for a very great length of time can it be assumed that part of the pseudoglobulin as well as of the albumin is precipitated in a denaturated state.

In every case, where a precipitate was produced by shaking, the latter has been found to be insoluble except in alkalis, which shows that denaturation has taken place.

Now the question arises what is the real cause of this denaturation of the proteins, which occurs when they are shaken with a gas, and what other processes accompany this shaking-effect.

The effect of shaking explained as a consequence of change in surface energy.

The observation that shaking of a protein solution with a neutral gas produces the separation of a solid body, can be traced back to 1851, when Melsens and Harting first observed this phenomenon, which was later confirmed by Smee (1863) and more closely investigated by Plateau (1873), who considered the formation of solid surface membranes to be of a similar nature. The same observations were made by Naegeli (1880) and Kauder (1886). To Metcalf (1905) we owe a thorough investigation of the nature of the formation of surface membranes, especially those occurring in solutions of peptone. Peptone as well as other substances, the solutions of which tend to form surface membranes, lower the surface tension of water [Freundlich, 1909]. According to Gibbs' thermodynamic principle those substances will be positively adsorbed at the free surface of the liquid, thus producing a relatively higher concentration in the surface. This increase of concentration in the surface may become so great, that gel-formation takes place. The conception of H. Freundlich (1909, p. 79) is probably more correct, namely that the adsorbed substance in the surface is undergoing reversible or irreversible alterations in a less soluble body, especially if the substance is by itself not readily soluble in water as the dispersion medium. This change in solubility has also been observed by Deveaux (1904), who noticed the formation of white insoluble membranes, when egg albumin was dropped on to a clean surface of water. These phenomena are especially well marked in the case of egg albumin and

have been closely investigated by Ramsden (1894). It is evident, that a soluble substance tending to form surface membranes, will show this tendency more so, if by means of shaking the surface is considerably enlarged. Now Ramsden demonstrated this accelerating effect of shaking on the formation of surface membranes, and he succeeded in bringing about almost complete coagulation of the protein content of an egg white solution by means of shaking.

What happens now in the case of a shaken serum? *In vivo* the serum has no free surface against air, but is everywhere surrounded by a wall covered with endothelium. It is probable that the adhesion on this wall is in equilibrium with the cohesion of the serum, thus giving rise to no surface tension of the serum and therefore thus insuring the stability of the serum proteins. If the serum has however a free surface against air, the cohesion will be larger than the adhesion to the air, and a certain positive value of surface tension of the serum against air will result. Now the serum is an electrolyte containing protein hydrosol, and its surface tension against air is less than that of pure water, as J. Traube (1908) first showed. This lower value of surface tension is chiefly due to the presence of proteins. If according to Maraghini (1912) the protein substances are removed by filtration through a collodium membrane, the filtrate will show a surface tension similar to water and even a little higher, this fact being due to the presence of neutral salts, which increase the surface tension. The proteins show therefore a tendency to be positively adsorbed on the free surface against air and the more they lower the surface tension of their dispersion medium, the greater is the tendency for this to occur. Comparative experiments have shown that the loss of surface tension produced by the serum albumins is found to be somewhat higher than that caused by the globulins.

I give below in a table some data, with regard to the surface tension of the different proteins. The figures represent the number of drops given by a stalagmometer of Traube and calculated as mentioned in my former paper (this *Journal*, 1913, 316).

From these data it follows that a serum albumin tends to lower the surface tension of water against air more than the globulins do. This is in accordance with experiments made by F. Bottazzi (1913), but is contrary to the findings of Iscovesco (1910), who found a pure egg albumin to increase the surface tension of water, whereas only the globulins cause a loss of surface tension. Either egg or serum albumin behaves differently as regards its influence upon surface tension, which

						Heated $\frac{1}{2}$ h. at 56°	Boiled
I.	Water	...	...	...	100.00	—	—
	Serum undiluted	...	...	...	111.65	—	—
	Serum diluted 1:8 with aqu. dist.	...	...	...	102.51	—	—
	Albumin fraction not rendered isotonic ($\frac{1}{8}$)	...	...	...	102.77	107.31	111.02
	„ „ rendered isotonic ($\frac{1}{10}$)	...	...	...	104.95	105.05	112.56
	Globulin fraction in 0.85 % NaCl ($\frac{1}{10}$)	...	...	...	102.16	104.95	106.70
	Serum dil. $\frac{1}{20}$ with 0.85 % NaCl	...	...	...	102.67	104.49	109.83
II.	Serum diluted 1:4 with aqu. dist.	...	...	...	...	...	102.50
	Albumin fraction not isotonic ($\frac{1}{4}$)	...	...	...	...	...	104.30
	„ „ „ heated $\frac{1}{2}$ hour at 56°	...	...	...	...	...	105.81
	„ „ „ 2 hours shaken at 37° and filtered	...	...	...	...	...	103.39
	„ „ „ Control 2 hours at 37°	...	...	...	...	...	105.06
III.	Serum diluted 1:10 with NaCl solution	...	...	...	...	...	103.67
	„ „ „ „ heated $\frac{1}{2}$ hour at 56°	...	...	...	...	...	106.87
	„ „ „ „ 2 hours shaken at 37°	...	...	...	...	...	103.67
	„ „ „ „ 2 hours control at 37°	...	...	...	...	...	104.19
	Globulin fraction, <i>well washed</i> with aqu. dist. then diluted 1:5 with 0.85 % NaCl						101.4
	Globulin fraction, heated $\frac{1}{2}$ hour at 56°	...	...	...	...	...	101.75
	„ „ 1 hour shaken	...	...	...	...	...	101.55
	Albumin fraction dil. 1:8, not isotonic	...	...	...	...	...	103.97
	„ „ heated $\frac{1}{2}$ hour at 56°	...	...	...	...	...	106.06
	„ „ 2 hours shaken at 37° and filtered	...	...	...	...	...	101.22
	„ „ Control at 37°	...	...	...	...	...	103.95

Surface tension of horse serum preparations.

						Aqu. dist.—100
1.	50 c.c.	aqu. dist.	+0.5 gr.	air dried whole horse serum	...	103.15
2.	50 c.c.	„	+0.5 gr.	„ albumin	...	113.74
3.	50 c.c.	„	+0.5 gr.	„ globulins (eu- + pseudoglob.)	...	107.08

would be very interesting, or the albumin is not free from the accidental presence of other substances, which lower the surface tension. These may be the serum pigments, which are naturally present in the CO₂-albumin fraction, and also in the albumin preparations obtained by salting out, for a total saturation with the sulphate of ammonium involves the pigments, these latter coming down with the precipitate. This question, viz. how far the observed loss in surface tension in albumin solutions is due to the actual accidental presence of such substances, I must leave open for the present, but for the sake of argument will assume it to be the case, namely that serum albumin produces a considerable loss of surface tension of the water against air.

Now a serum exposing a free surface to air is by that reason alone

rendered unstable, involving as it does the adsorption of albumins. This adsorption decreases the protecting influence which the albumins exert on the dispersity of the euglobulins in virtue of their greater influence on surface tension. This process is favoured by any increase of temperature which produces an increase in the intensity of the Brownian movement. The euglobulin thus becoming more labile tends to aggregation and precipitation, undergoing a slowly but steadily progressing denaturation. This process, which takes place when serum is exposed with a free surface to air, and which is accelerated by an increase of temperature, is accelerated to a considerable extent when, by means of shaking with air, not only is its free surface enlarged but also continuously changed and renewed. This continuous alteration of the surface causes the already aggregated protein particles to disperse again, which process, when continuously repeated, favours the condition of irreversible insolubility of the proteins, as has been already pointed out by Ramsden. The dispersion of already aggregated proteins is the more marked, the higher the protein concentration in the serum is, *i.e.* the more the mutual protecting influence of the different proteins can be exerted. Dilution of the serum favours therefore the occurrence of the condition of coagulation and irreversible insolubility. This irreversible change in solubility affects naturally first the euglobulins, which by themselves show the least tendency to solution. If the serum has been previously heated, which causes a considerable loss of surface tension, as observed by Traube (1908) and confirmed by many other authors, it may be possible to assume that the formation of capillary-active substances by heat, according to L. Berczeller (1913), albumoses and pepton, prevents the proteins from getting adsorbed on the surface, thus allowing them a greater stability against the influence of storage and shaking. This may also be taken into account when considering the influence of the electric charge of the denaturated protein particles upon their state of dispersion.

In a former paper of mine (1913) I was able to show that the surface tension of a serum is increased by shaking. The collected froth shows however lower surface tension than the shaken liquid, which is in accordance with Gibbs' principle.

How far the shaking effect upon a serum is due to alterations of the surface energy is best demonstrated by the fact that the addition to a serum of traces of such a capillary-active substance, as saponin is, completely prevents the proteins from being affected by the shaking, whatever the length of time during which it is shaken.

The possibility that, apart from alterations of the surface energy, other factors may play a more or less important part in the production of the shaking effect, cannot be excluded. It may be assumed, for instance, that the rapid movement of the air bubbles through the liquid gives rise to an electric charge on their surface, the density of which will increase with the curvature of the bubbles, *i.e.* with their smallness, and it may be possible that these electrically charged surfaces play a part in favouring the aggregation and coagulation of the proteins in solution. No definite evidence however is forthcoming to support this view.

Finally I mention the probability that a change in the H⁺-concentration in the shaken serum has taken place. Using neutral red and rosolic acid as indicators, I could not find however any difference in reaction of a shaken as compared with an unshaken serum. The addition of either traces of alkali or of acid to a serum proved to be without any inhibiting effect upon the effect of shaking, but I found that alkali produced a slight acceleration of the coagulation and precipitation in the shaken serum. In view of the importance of the H⁺-concentration of a serum as regards its serological properties, the question of any alteration of H⁺-concentration as a result of shaking deserves a special study.

GENERAL CONCLUSIONS.

Shaking a serum with air produces coagulation and denaturation of the euglobulin, due to alterations in the surface energy of the serum, leading to adsorption of albumin, thus rendering the euglobulins labile, which then coagulate and become insoluble.

This process is not due to oxidation, and it has been shown that conditions which render the euglobulin more stable, such as previous heating and the presence of a hypertonic medium, tend to inhibit the effect of shaking, whereas dilution with water or storage, rendering the euglobulins labile, favours also the effect of shaking.

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STUDIES ON THE INACTIVATION OF COMPLEMENT BY SHAKING.

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INTRODUCTION.

IN a former paper on the inactivation of the haemolytic complement by means of shaking (1913), I considered it possible that the process of shaking-inactivation might be explained by assuming that an adsorption of the complement occurred on the surfaces of the precipitate, which is always found when a serum is shaken. I was unable however either to fix the complement of a fresh serum by adding the centrifuged coagula of a shaken serum or, on the other hand, to set free a complement presumably adsorbed on the surface of precipitated proteins, which are found to be soluble only in alkali. I therefore had to leave undecided the question as regards the nature of the process of shaking-inactivation.

About the same time and independently P. Schmidt and M. Liebers (1913), working on the shaking-inactivation of the haemolytic complement, came to the following conclusion: shaking of guinea-pig serum produces precipitation of the euglobulins followed by adsorption of the complement on these newly-formed surfaces. The visibly coagulated globulin is only a minimal part of the total globulin, and the shaken and centrifuged serum contains therefore not only globulins but also complement not yet adsorbed. They succeeded in bringing the globulin, precipitated by shaking, back into solution by a normal E-piece, thus setting free the complement adsorbed on these surfaces, enabling it to act again.

L. Hirschfeld and R. Klinger (1914), in their researches on the shaking-inactivation, worked with human serum and came to the conclusion that shaking produces alteration in the stability of the globulins, thus causing a part of them to coagulate. This alteration interferes

with complement action, and sera so treated become anti-complementary, giving for instance a positive Wassermann reaction. Recent work of A. Bessemans (1913), however, shows that human and guinea-pig sera are different as regards the stability of their globulins, and results, obtained with experiments on human serum, are therefore not to be compared with those obtained with guinea-pig serum except to a restricted degree.

I therefore resumed my investigations on the nature of the inactivation process by shaking chiefly with a view to ascertain in how far the conception of P. Schmidt could be adapted to the experimental facts.

I give below a record of experiments made from a serological point of view as regards the influence of shaking upon the haemolytic complement of guinea-pig serum, and consider them to be a continuation of my former work on the same subject.

Technique of experiments.

The technique employed in shaking the sera is principally the same as that indicated in my former paper (1913). Haemolysis was due to the complementing action of the serum in combination with sensitized sheep red corpuscles, of which a 2·5 % emulsion in 0·85 % saline was used. Sensitization was effected with inactivated rabbit immune-serum, the dose of which will be given in the protocols. The following scheme has been adopted to illustrate different degrees of haemolysis :

- ☐ No haemolysis.
- ☒ Faint trace of haemolysis.
- ☐ Very slight haemolysis.
- ☐ Well-marked haemolysis.
- ☐ Half haemolysed.
- ☐ Strong haemolysis.
- ☐ Very strong haemolysis.
- ☒ Nearly complete haemolysis [a slight trace of unlysed cells at the bottom].
- ☒ Complete haemolysis.

Experimental part.

In another communication (1914), considering the effect of shaking upon a serum from a more general standpoint, I have shown that the effect of shaking upon a serum is due to alterations in the surface energy

involving changes of the serum albumins and denaturation of the serum proteins, which first of all affects the euglobulin. The process is to be considered as progressing with the time, till irreversible denaturation of the proteins is effected. The various stages of this process are serologically characterised by the steadily progressing loss of complement activity as indicated by the different procedures necessary to restore the complementing power. The different factors necessary to the restitution of complement activity have been already mentioned in my former paper (1913), but before I can attempt to explain these very complicated relations, I must first of all complete the description of my experiments.

Reactivation of a shaken serum by a fresh serum.

A serum inactivated by shaking can be reactivated by the addition of fresh serum. This reactivation can be effected under any conditions, and it is the better marked the more the shaken inactivated serum can be reactivated by other factors, as will later be shown. The question as to the restitution of a shaken serum by a normal serum is, in reality, how far the former interferes with the action of normal complement. Jakoby and Schuetze (1910) found no inhibition to take place, a fact which I can confirm. It proved to be the same, whether the precipitated globulins were removed by the centrifuge and filtering, or not removed at all. I found also that the precipitate itself showed no inhibiting effect on normal complement action. Only when present in large excess did slight inhibition sometimes occur. This is confirmed by the work of Landsteiner and Stankovic (1906), who found that heat-coagulated serum proteins produced a slight inhibiting effect. Hirschfeld and Klinger (1914), however, found that sometimes the centrifuged precipitate of a shaken serum exerted an anti-complementary effect, but this was not constant. On the other hand the sera after shaking were sometimes so anti-complementary as to give a positive Wa. reaction, even if the serum had been well centrifuged. But it must be remembered that these authors worked exclusively with human serum, which may behave very differently from guinea-pig serum when shaken, the latter showing no inhibiting effect after shaking, as also Courmont and Dufourt found (1912).

Whether inhibition of normal complement action takes place or not would according to P. Schmidt's conception be explained by assuming a partial or complete saturation of the adsorbing surface of the

precipitated globulins, but there seems to be some difficulty in understanding why the albumins of the fresh serum do not effect a solution of the globulins, thus setting the adsorbed complement free, such as occurs on the addition of a normal E-piece. It may be possible also to assume, that addition to a fresh serum of such a dispersed phase as an opalescent shaken serum represents gives rise in the fresh serum to adsorptions by increase of surface analogous to those which effected the inactivation by shaking.

The reactivation of a shaken serum by means of the complement fractions.

As regards the technique it must be mentioned, that in order to obtain the complement fractions, the CO_2 -method of Liefmann has been employed, it giving more uniform results than the HCl -method of Altmann, which I had previously employed. After diluting the serum 1 : 8 with aqu. dist., CO_2 was passed through for half an hour, the temperature being kept low by means of ice. The serum was then well centrifuged, the supernatant fluid filtered through hardened paper and then rendered isotonic. The precipitate was washed once with distilled water and diluted with 0.85 % NaCl solution immediately before use.

For the sake of brevity I shall employ the following signs :

C_n = normal complement containing serum.

C_{sh} = serum inactivated by shaking at 37°C .

C_{th} = serum inactivated by heating at 56°C . for half an hour.

C_5 , C_{10} , etc. = sera inactivated by heating at 56°C . for 5, 10, etc. minutes.

E_n and M_n = CO_2 fractions obtained from a normal serum (C_n).

E_{sh} and M_{sh} = CO_2 fractions obtained from a shaken inactivated serum (C_{sh}).

E_{th} and M_{th} = CO_2 fractions obtained from a thermo-inactivated serum (C_{th}).

E_5 , E_{10} , etc. and M_5 , M_{10} , etc. = CO_2 fractions from sera heated at 56°C . for 5, 10, etc. minutes.

Ritz (1912) showed that the process of inactivation by shaking consists of two stages. In the first stage it is possible to reactivate C_{sh} by M_n or E_n , in the second stage this has become impossible. I found previously the reactivation by M_n to be impossible, and if we are to understand by reactivation a complete restitution of an

otherwise completely inactivated shaken serum, I must still adhere to that opinion; but that a partial reactivation can be effected by M_n is to be seen from the experiments I and II.

Exp. I. Guinea-pig serum, diluted 1 : 10 with 0.85 % saline, was shaken for different times, then tested as regards its haemolytic power and its reactivation by M-piece and E-piece.

10 c.c. of this serum was shaken in tube of 60 c.c. volume.

Haemolysis of 0.5 c.c. sensit. red cells (AB dose = three times the single lysing dose) after 1 hour at 37° and 15 hours at room temperature.

	+ C_{sh}	0.5 c.c. E_n 0.5 c.c.	0.5 c.c. M_n 0.5 c.c.	0.5 c.c. saline 0.5 c.c.
after being shaken at 37° for $\frac{1}{2}$ hr.				
1 hr.				
1½ hrs.				
2 hrs.				
+ M_n ...				
+ E_n ...				
+ C_n control serum				
+ 0.85 % saline ...				

Exp. II. C_n diluted $\frac{1}{10}$ with saline was shaken; 13 c.c. liquid in 67 c.c. containing tube.

Hours shaking time		$\frac{1}{10} C_{sh}$			0.5 c.c. of							
		1.0	0.5	0.25	M_n	E_n	C_5	C_{10}	C_{15}	C_{20}	C_{25}	C_{30}
C_{sh}	$\frac{1}{2}$											
	1											
	1½											
	2											
	2½											
	3½											
C_n	...											
C_n control	3½ h. at 37°											
	+ 0.5 c.c. E_n											
	+ 0.5 c.c. M_n											

Every half an hour 1.5 c.c. was taken out, centrifuged and tested.

C_n was undiluted, exposed to 56° C. for between 5 and 30 minutes, then diluted 1 : 10 with saline.

(AB dose and amount of red cell emulsion equal to Exp. I.)

Haemolysis after 1 hour at 37° and 15 hours at room temperature.

To 0.5 c.c. of C_{sh} was added 0.5 c.c. of M_n , E_n or C_n , the total volume being 1.5 c.c.

These experiments I and II, as well as many others giving similar results, demonstrate that a serum, the complementing power of which is considerably diminished by the shaking, but which is yet not completely inactivated, can be fully restored by M_n . If however the inactivation process is further advanced and finally completed, the haemolytic action of the serum can no longer be restored by M_n , except to a very small degree, while E_n has still its full reactivating effect. Further shaking of the serum will diminish and finally abolish the possibility of reactivation by E_n , and, as Exp. II shows, also that of C_{th} . Therefore the inactivation process by shaking is to be considered as progressing in such a manner that the possibility of reactivation by M_n is first lost, later that of E_n , and finally the reactivation by C_{th} disappears. Such a serum is then irreversibly inactivated.

The reactivation of a serum inactivated by shaking, by means of a thermo-inactivated serum and the relation of this process to the reactivation by the normal complement fractions.

The reactivation of C_{sh} by C_{th} is in some way connected with the reactivation by means of M_n and E_n , but before attempting to explain this, I will give the results of a series of experiments demonstrating these facts.

The above-mentioned Exp. II shows that the possibility of reactivating C_{sh} by C_{th} decreases progressively, either if the C_{th} is heated for a longer and longer time, or if the C_{sh} is submitted to a more prolonged shaking.

In a former paper (1913) I pointed out that, generally speaking, C_{sh} can be reactivated by C_{th} as long as the latter can be reactivated by E_n . I shall refer to this later. In Exp. II however this is only approximately the case, for it is shown that C_{15} is able to reactivate a C_{sh} shaken 2 hours, to a large extent, in spite of the fact that neither E_n nor M_n has any effect on C_{th} . This is exactly what Ritz explains by assuming a third component.

Exp. III. A guinea-pig serum of 24 hours standing was partly shaken at 37° undiluted, partly heated at 56° for half an hour and partly not treated.

All sera were then submitted to the effect of CO_2 and thus the fractions were obtained, the signs for which are employed as above stated.

Haemolysis of 1 c.c. sensit. red cell emulsion after 1 hour at 37° and 15 hours at room temperature.

(AB dose = four times the single lysing dose.)

Exp. III shows that C_{sh} can be reactivated by M_n , the more so the greater the dilution of M_n , and that M_{30} , i.e. a M-piece derived

	c.c.	1.0	0.5	0.25	0.15	0.1	0.0
$\frac{1}{10}$ C_n							
„ C_{sh}							
„ C_{30}							
1 c.c. $\frac{1}{10}$ diluted E_n + 1 c.c. 0.85 % saline							
„ $\frac{1}{20}$ „ M_n + „ „ „							
„ $\frac{1}{10}$ „ E_n + „ $\frac{1}{20}$ diluted M_n							
„ „ „ E_{30} + „ 0.85 % saline							
„ „ „ M_{30} + „ „ „							
„ „ „ E_{30} + „ $\frac{1}{10}$ diluted M_{30}							
„ „ „ E_{sh} + „ 0.85 % saline							
„ „ „ M_{sh} + „ „ „							
„ „ „ E_{sh} + „ $\frac{1}{10}$ diluted M_{sh}							
1 c.c. $\frac{1}{10}$ diluted E_n + 1 c.c. $\frac{1}{10}$ diluted M_{sh}							
„ „ C_{30} + „ „ E_n							
„ „ C_{30} + „ „ C_n							
„ „ C_{30} + „ „ C_{sh}							
„ „ C_{30} + „ „ E_{sh}							
„ „ C_{30} + „ „ M_{sh}							
„ „ M_n + „ „ E_{sh}							
„ „ E_n (heated) + „ „ M_{sh}							

1 c.c. $\frac{1}{16}$ diluted C_{sh}	+ 1 c.c. $\frac{1}{2}$ diluted M_n	...	
" "	" + " $\frac{1}{4}$ " "	...	
" "	" + " $\frac{1}{8}$ " "	...	
" "	" + " $\frac{1}{2}$ " M_{sh}	...	
" "	" + " $\frac{1}{4}$ " "	...	
" "	" + " $\frac{1}{8}$ " "	...	
" "	" + " $\frac{1}{2}$ " M_{30}	...	
" "	" + " $\frac{1}{4}$ " "	...	
" "	" + " $\frac{1}{8}$ " "	...	
" "	" + " $\frac{1}{16}$ " E_n	...	
" "	" + " $\frac{1}{32}$ " "	...	
" "	" + " $\frac{1}{64}$ " "	...	
" "	" + " $\frac{1}{16}$ " E_{sh}	...	
" "	" + " $\frac{1}{32}$ " "	...	
" "	" + " $\frac{1}{64}$ " "	...	
" "	" + " $\frac{1}{16}$ " E_{30}	...	
" "	" + " $\frac{1}{32}$ " "	...	
" "	" + " $\frac{1}{64}$ " "	...	
" "	" + " $\frac{1}{16}$ " E_n (heated)	...	

from a thermo-inactivated serum, gives a more complete reaction than M_n , M_{sh} having no effect.

In the case of the reactivation by E-piece it is irrelevant whether the E-piece is derived from a normal or from a thermo-inactivated serum. Even the addition of E_{sh} has a marked effect.

The thermo-inactivated serum, C_{30} , was not anti-complementary nor could it be reactivated by E_n or by E_{sh} , except to a very slight extent. On the other hand its reactivating power on C_{sh} was well marked, the latter being however not completely inactive.

Exp. IV. Fresh guinea-pig serum was treated with CO_2 , and the M-piece thus obtained shaken for 1 hour at 37° ; other parts of the M-piece were diluted in different concentrations and then either heated or not treated otherwise.

Each tube contains 1 c.c. $\frac{1}{10}$ diluted C_n , M-piece in decreasing amounts, saline to fill up to 2 c.c. and 1 c.c. sensit. red cell emulsion. (AB dose = three times the single lysing dose.)

Haemolysis after 1 hour at 37° and 24 hours standing at room temp.

The control reactions were satisfactory.

				c.c.	1.0	0.5	0.25	0.15
M_n 1:2 diluted	...	...	...					
„ 1:5 „	...	...	...					
„ 1:10 „	...	...	...					
„ 1:20 „	...	...	...					
$\frac{1}{10}$ diluted M_n 1 hr. shaken at 37°	...							
M_n 1:5 diluted, heated 30 mins. at 56°								
„ 1:10 „ „ 10 „ „								
„ „ „ „ 20 „ „								
„ „ „ „ 30 „ „								
„ 1:20 „ „ 30 „ „								

Exp. IV shows the anti-complementary effect of a normal M-piece on a normal serum, decreasing with dilution and with heating, the shaken M-piece having no longer any anti-complementary effect.

Exp. V. Haemolysis after 1 hour at 37° and 15 hours standing at room temp.

1 c.c. sensit. red cell emulsion, AB dose being three times the single lysing dose.

Total volume in each tube = 3 c.c., of each factor 1 c.c. being in reaction.

From Exp. V it follows that a shaken serum which is completely inactivated and unable to be reactivated by a normal M-piece is restored by the addition of a M-piece obtained from a thermo-inactivated serum.

The collected froth of a shaken serum retains its haemolytic power longer than the shaken liquid.

No difference is to be found in the reactivation of a shaken serum by an E-piece, whether obtained from a normal serum or from a thermo-inactivated serum.

In the course of thermo-inactivation the property of being reactivated

1 : 10 diluted E _n	+	saline	...	
.. M _n	+	..	...	
.. E _n	+	M _n $\frac{1}{10}$ diluted		
.. E ₅	+	saline	...	
.. M ₅	+	..	...	
.. E ₅	+	M ₅ $\frac{1}{10}$ diluted		
.. E ₃₀	+	saline	...	
.. M ₃₀	+	..	...	
.. E ₃₀	+	M ₃₀ $\frac{1}{10}$ diluted		
.. M _n	+	E ₅		
.. ..	+	E ₃₀		
.. E _n	+	M ₅		
.. ..	+	M ₃₀		
.. C _n	+	M _n $\frac{1}{2}$..		
.. ..	+	M ₅		
.. ..	+	M ₃₀		
.. C _{sh}	+	M _n		
.. ..	+	.. $\frac{1}{10}$..		
.. ..	+	M ₅ $\frac{1}{2}$..		
.. ..	+	.. $\frac{1}{10}$..		
.. ..	+	M ₃₀ $\frac{1}{2}$..		
.. ..	+	.. $\frac{1}{10}$..		
.. ..	+	saline	...	
.. collected froth of C _{sh} +		..	...	
.. C _{sh}	+	E _n $\frac{1}{10}$ diluted		
.. ..	+	E ₅		
.. ..	+	E ₃₀		

by the normal complement fractions is lost quicker in the case of the M-piece than in the case of the E-piece.

The anti-complementary effect produced on normal complement by

a M-piece obtained from a thermo-inactivated serum is very small when compared with the inhibiting effect of a normal M-piece.

Exp. VI. Haemolysis after 1 hour at 37° and 15 hours at room temperature.

1 c.c. sensit. red cell emulsion. AB dose = three times the single lysing dose.

Total volume in each tube = 3 c.c., of each factor 1 c.c. being present.

						C _{sh}	E _n	Saline
1 : 5	diluted	M _n	...	...	...			⋮
1 : 10	„	„	...	...	...			
1 : 20	„	„	...	...	...			
1 : 5	„	M _n heated 10 mins. at 56° C.						⋮
1 : 10	„	„	„	„	„			
1 : 20	„	„	„	„	„			
1 : 5	„	M ₁₀	...	...	...			⋮
1 : 10	„	„	...	...	...			
1 : 20	„	„	...	...	...			
1 : 5	„	M ₁₀ heated 10 mins. at 56° C.						⋮
1 : 10	„	„	„	„	„			
1 : 20	„	„	„	„	„			
1 : 10	„	E _n	...	...	...			⋮
„	„	E _n heated 10 mins. at 56° C.						
„	„	E ₁₀	...	...	...			
„	„	E ₁₀ heated 10 mins. at 56° C.						
„	„	E _n	...	...	...			
„	„	E ₁₀	...	...	...			
„	„	C _n	...	...	...			
„	„	C ₁₀	...	...	...			
„	„	C _{sh} at 37°	...	...	...			
„	„	C control at 37°	...	...	...			

This experiment shows first of all the great difference in the action of a M-piece upon an E-piece or on a shaken inactivated serum. Exp. VI shows also what we have already seen in Exp. V, that a shaken inactivated serum, not being reactivated by a normal M-piece, will become so, if the M-piece is derived from a thermo-inactivated serum, exposed to 56° C. for 10 minutes. Such a M-piece has a slight effect, when combined with a normal E-piece, but while heating such a M-piece will completely destroy this effect, the action of it on a shaken serum is but slightly diminished.

The same experiment shows further, that the effect of heating an isolated E-piece will not altogether destroy its power of reactivating a shaken serum, but it will do so, if the E-piece has been obtained from a serum already heated for 10 minutes at 56° C.

It is of interest to note in this connection, that Exp. III shows that a M-piece obtained from a shaken but not completely inactive serum acted quite well with a normal E-piece, but did no longer do so if the latter had been heated.

Exp. VII. Haemolysis of 1 c.c. sensit. red cell emulsion (AB dose = three times the single lysing dose) after 1½ hours at 37°.

	$\frac{1}{10}$ diluted E_n , c.c.	1.0	1.0	1.0	1.0	0.0
	0.85 % saline, c.c.		0.5	0.75	0.85	1.0
	+ c.c. ...	1.0	0.5	0.25	0.15	1.0
$\frac{1}{10}$ diluted M_n (of C_n)	...					
" " M_n shaken at 37°	...					
" " M_n control at 37°	...					
" " M_{sh} (of C_{sh})	...					
" " M_n (of C control)	...					
	$\frac{1}{10}$ diluted M_n , c.c.	1.0	1.0	1.0	1.0	0.0
	0.85 % saline, c.c.		0.5	0.75	0.85	1.0
	+ c.c. ...	1.0	0.5	0.25	0.15	1.0
$\frac{1}{10}$ diluted E_n	...					
" " E_n shaken (isotonic) 37°	...					
" " E_n " (not isotonic) 37°	...					
" " E_n control (isotonic) 37°	...					
" " E_n " (not isotonic) 37°	...					
" " E_{sh} (of C_{sh})	...					
" " E_n (of C control)	...					

0.85 % saline, c.c.				0.5	0.75	0.85	0.9	
+ c.c. ...				1.0	0.5	0.25	0.15	0.1
$\frac{1}{10}$ diluted C_n	...	...	...					
„ „ C_{sh} at 37°	...	...	...					
„ „ C control at 37°	...	...	...					

From the observations made in Exp. VII the following conclusions can be drawn :

The normal action of a M-piece on an E-piece is abolished by shaking the isolated M-piece. If the M-piece however is derived from a shaken serum, the complementing power of which is considerably weakened by shaking, such a M-piece still has a well-marked effect with a normal E-piece.

On the other hand, if the action of a normal E-piece on a normal M-piece is considered, no loss of activity of the E-piece is observed when the latter has been shaken for an equal length of time as the M-piece.

[Only shaking the E-piece for a very long time will destroy its action.] The E-piece of a shaken serum is altered only to a very slight extent.

Exp. VIII. Fresh guinea-pig serum $\frac{1}{10}$ diluted was shaken 4 hours at 37° , then centrifuged and submitted to the following tests :

Haemolysis of 0.5 c.c. sensit. red cell emulsion (AB dose = twice the single lysing dose) after 1 hour at 37° and about 15 hours at room temperature.

$E_n B$ = E-piece, which after being rendered isotonic, was filtered through a Berkefeld filter (40×14 mm.).

$E_n A$ = E-piece, the reaction of which has been rendered equal to that of the C_n by means of addition of $\frac{n}{250}$ NaOH, neutral red and rosolic acid being used as indicators.

From Exp. VIII it follows that the reactivation of a shaken inactive serum by means of an E-piece is only slightly diminished if the latter has been filtered through a Berkefeld filter. [This observation is not constantly found, cf. Exp. IX.] But the action of the E-piece on a M-piece is totally abolished by the Berkefeld filtration.

A normal E-piece reactivates a shaken serum even after being heated 15 minutes, but a slight addition of alkali to the E-piece destroys its action, whether heated or unheated, on a shaken serum as well as on a normal M-piece [not constantly found].

1 c.c. $\frac{1}{10}$ diluted C _{sh}	+	2 c.c. $\frac{1}{10}$ diluted E _n	..	...	...	...	■
..	..	..	+ 1 ..	..	E _n ..	...	■
..	..	..	+ 2 ..	..	E _n B	...	■
..	..	..	+ 1 ..	..	E _n B	...	■
..	..	..	+ 2 c.c. $\frac{1}{20}$	..	M _n ..	...	□
..	..	..	+ 1 ..	..	M _n ..	...	□
..	..	..	+ 2 c.c. $\frac{1}{10}$	..	E _n heated for 15 mins. at 56° C.	...	■
..	..	..	+ 2 ..	..	E _n A ..	..	□
..	..	..	+ 2 ..	..	C _n ..	..	■
..	..	..	+ 2 ..	..	E _n A	...	□
..	..	..	+ 1 ..	..	E _n A	...	□
1 c.c. $\frac{1}{20}$ diluted M _n	+	1 c.c. $\frac{1}{10}$ diluted E _n	...	...	...	...	■
..	..	..	+ 1 ..	..	E _n B	...	□
..	..	..	+ 1 ..	..	E _n A	...	□
..	..	..	+ 1 ..	..	E _n heated for 15 mins. at 56° C.	...	□
..	..	..	+ 1 ..	..	E _n A ..	..	□
1 c.c. $\frac{1}{10}$ diluted C _{sh}		+ 1 c.c. saline	...	...	...	...	□
..	..	C _n	+	..	...	..	■
..	..	C _n (15 mins. at 56°)	+	..	..	...	□

Haemolysis of 0.5 c.c. sensit. red cell emulsion after 1 hour at 37° and 15 hours at room temperature; sensitization effected with three times the single lysing dose of AB.

Exp. IX shows that a shaken serum can no longer be reactivated by an E-piece, if submitted to Berkefeld filtration. Contrarily to Exp. VIII this experiment shows that the E-piece after Berkefeld filtration was no longer capable of reactivating a shaken serum.

If an E-piece is heated for too long a time its action on a shaken serum will disappear.

Addition of alkali to an E-piece has a somewhat lesser inhibiting effect in this experiment than in Exp. VIII.

A thermo-inactivated serum, in spite of being reactivated by an E-piece, showed no action on the shaken inactive serum.

Experiment IX.

$E_n A = 0.5$ c.c. $E_n + 0.3$ c.c. $\frac{n}{250}$ NaOH in 0.85 % NaCl in each tube.

$E_n A_{th} = 0.5$ c.c. $E_n + 0.3$ c.c. $\frac{n}{250}$ NaOH in 0.85 % NaCl, the mixture heated for 30 mins. at 56° C.

NaCl = 0.5 c.c. NaCl solution + 0.3 c.c. $\frac{n}{250}$ NaOH in 0.85 % NaCl in each tube.

	E_n	$E_n B$	$E_n A$	E_{th}	$E_n A_{th}$	C_{th}	M_n	NaClA	NaCl
0.5 c.c. $C_{sh} \frac{1}{10}$ diluted, 1 hr. shaken, cloudy, being not filtered									
0.5 c.c. $C_{sh} \frac{1}{10}$ diluted, 2 hrs. shaken, not filtered (cloudy)									
0.5 c.c. $C_{sh} \frac{1}{10}$ diluted, 2 hrs. shaken, filt. through hard paper (opalesc.)									
0.5 c.c. $C_{sh} \frac{1}{10}$ diluted, 2 hrs. shaken, filt. through Berkef. filt. (clear)									
0.5 c.c. $C_{sh} \frac{1}{10}$ diluted, 3 hrs. shaken, not filtered									
0.5 c.c. $M_n \frac{1}{10}$ diluted									
0.5 c.c. C_{th} (30 mins. at 56°) $\frac{1}{10}$ diluted									
0.5 c.c. C control serum $\frac{1}{10}$ diluted, (3 hours at 37°)									

Summary of the experimental results.

In order to better survey the results obtained in the experiments described, I summarise them as follows :

A shaken serum can be reactivated by M_n , E_n , C_{th} or not at all, according to the degree of inactivation produced by the shaking [I, II].

The restitution effect of M_n is only found if the activity of the serum has not yet been completely destroyed [I, II]. This action of M_n disappears when the isolated M_n has been shaken or heated [VI], but a M-piece of a C_{th} may still exert full reactivation on a C_{sh} , when M_n no longer produces such an effect [V, VI]. Even heating a M_{th} will influence but little its restitution effect on a C_{sh} [VI]. Normally M_n has an anti-complementary effect upon C_n [IV]. This effect will be diminished by increasing dilution of the M_n and with the influence of heat upon it, but it will altogether disappear if M_n has been shaken [IV]. The normal action of M_n upon E_n will also be destroyed either by shaking or heating of the M_n [VII]. A M_{th} however still exerts some action upon E_n , which promptly disappears, if such an isolated M_{th} is exposed to heat. But even then it retains some of its reactivating power on a C_{sh} [VI].

A shaken serum completely inactivated as regards complement action can still be reactivated by E_n or C_{th} , when M_n will no longer have any effect [I, II]. It requires a much longer time to render an E_n inactive by shaking [VII] than it does for a M_n . An E_n works equally well as regards reactivation of C_{sh} , whether obtained from a C_n or C_{th} [V, VI]. If an isolated E_n be heated, its power of reactivating a shaken inactive serum is maintained for a longer time than its activity with a M_n [VIII]. The addition of alkali to an E_n , which obtained by CO_2 is acid in character, tends to inhibit its restitution effect on C_{sh} , but this result has so far not been obtained with regularity.

The reactivation of C_{sh} by means of C_{th} can take place, in spite of C_{th} not being able to be reactivated by M_n or E_n . The property of C_{th} to reactivate a C_{sh} decreases with a prolonged exposure to heat. *Vice versa*, however, a C_{th} may be reactivated by an E_n and yet be without effect on C_{sh} [IX]. In such a case no anti-complementary effect of C_{th} on a normal C_n could be observed [II].

By means of Berkefeld filtration it is possible to render a C_{sh} no longer able of being reactivated by E_n [IX], and on the other hand to allow an E_n still to retain its action on C_{sh} [VIII].

Theoretical part.

First of all I think it certain that shaking produces a progressing alteration of all the serum proteins, leading finally to their denaturation. This alteration affects principally and first of all the euglobulin, changes in the albumin and pseudoglobulin following later, but always being present before visible coagulation takes place. There is not yet enough evidence to identify the complementing property of a serum with a certain definite state of equilibrium in the physical conditions of the different proteins, as regards their quantity and their degree of dispersity, which latter are closely connected with the amount of salts present and the H^+ -concentration. But it can be safely assumed that complement action, whether due to an existing substrate or not, is dependent on certain physical conditions of the serum and will therefore become lost as a consequence of anything which alters these conditions. The reactivation of a serum is only a question of how far these alterations are reversible or not.

Now, the following explanation may cover at least the majority of observations, if not all.

Addition of a M-piece to a shaken serum can only produce reactivation if the alteration, which the albumins undergo by shaking, is not yet so far advanced as to prevent them from controlling the dispersity of euglobulin. It is evident that irreversible changes in the added M-piece brought about by shaking or heating destroy any effect which the addition of a M-piece may otherwise have. Alterations in the albumins in a serum produced by shaking take place more rapidly than the denaturation of the euglobulin, which latter process is only the consequence of the former, as I explained in greater detail in another communication [1914].

From this it follows that reactivation by M-piece very soon disappears, and that addition of an E-piece has an effect only so long as there are euglobulins still in a reversible condition of commencing denaturation. Their removal by a Berkefeld filtration abolishes any reactivation by an E-piece, but the Berkefeld filtration of an E-piece is not necessarily followed by a loss of its restitution power, for I found that Berkefeld filtration chiefly retains the pseudoglobulin of the E-piece (1914). Such a Berkefeld filtered E-piece shows no longer any activity with a normal M-piece, which is difficult to explain. This may be due to the absence of pseudoglobulins, which according to the recent work of Browning and Mackie (1914) represent the main factor in the haemolytic complementing action of a serum. I could however not obtain these results with well dialysed preparations of the different proteins of guinea-pig serum, so I must leave this question undecided. On the other hand, I found that addition of pure albumin obtained by fractionated salting out with the sulphate of ammonium has a similar but weaker effect on a shaken inactive serum than that of an E-piece [not constantly found]. To destroy this power of E-piece by shaking it requires a longer time, owing to its solubility in water being greater than that of the euglobulin.

If the shaking effect however is so advanced that no more euglobulin is left in a reversible state, the addition of both, E-piece as well as pure albumin, will have no more effect. If this be the case, sometimes a thermo-inactivated serum is found to have still a marked effect on the reactivation of a shaken serum, but before attempting to explain this fact the alterations of a fresh serum produced by heat must be considered.

As I described in greater detail in another communication, heating a serum to 56° C. for half an hour is followed by more or less marked denaturation of its proteins [H. Chick and C. J. Martin, 1913]. The H⁺-concentration is decreased and the heat denaturated particles are

kept in dispersion by their negative charge. If by acidification with CO_2 the particles become isoelectric with the medium, they aggregate and form a precipitate. The precipitate thus obtained (M_{th}) contains not only euglobulin with some pseudoglobulin, which form the precipitate by CO_2 in the case of a normal serum diluted with water, but it contains that portion of all proteins which has been affected by the heat. In redissolving the precipitate in saline, a part of it is often found to be insoluble, probably consisting chiefly of euglobulin. Such a M_{th} due to its albumin content can still reactivate a shaken serum upon which a normal M-piece will no longer have any effect. Perhaps it is the electrical charge of its particles and also its albumin content which renders such a M_{th} more resistant to heat than a normal M-piece.

If a serum is exposed to 56°C ., its complementing power rapidly decreases with the length of time, and this happens a little sooner in the case of an undiluted serum, due probably to the ratio of the protein content to the salt concentration being higher in the case of a diluted serum, and to the fact that the presence of salts lower the coagulation rate by heat [Chick and Martin].

If the time of exposure to 56°C . be varied, and the different samples of sera so obtained be treated by CO_2 in order to obtain the M-piece or E-piece, it is found by their combination with the corresponding fractions of a normal serum [cf. Exp. V] that first the E-piece, later the M-piece, is destroyed, *i.e.* that an action of a M_{th} in combination with an E_n can be obtained for a greater length of time than the action of an E_{th} with a M_n . This has been already found by many other authors [Sachs, 1913]. From this it follows that as long as a shaken inactivated serum can be reactivated by a normal E-piece, it can also be reactivated by a thermo-inactivated serum, if the latter is capable of being reactivated by an E-piece. In some cases however such a thermo-inactivated serum is found to have no influence on a shaken inactivated serum, in spite of both being reactivated by a normal E-piece. I think that alterations in the H^+ -concentration account for this phenomenon.

On the other hand, a thermo-inactive serum, incapable of being restored by an E-piece, may still reactivate a shaken serum. I assume that such a thermo-inactivated serum may have the faculty of bringing even the denaturated euglobulins back into solution, perhaps owing to its alkalinity. A similar faculty of lysing a precipitate is attributed to a heated serum by Welsh and Chapman (1909) in explaining their observation of the inhibition which a heated serum exerts on specific precipitation. If however the denaturation of the shaken proteins has

advanced too far, even this lysing effect of a heated serum can no longer be obtained.

There are other observations more difficult to explain, for instance the fact that a M-piece of a serum, heated for 10 minutes at 56°C ., gives a better reaction with a shaken serum than with a normal E-piece [Exp. VI], and further, that 10 minutes heating of such a M_{th} will abolish the effect on an E-piece but only slightly diminish the action with a shaken serum. I think it very probable that here again alterations in the H' -concentration play the most important part. Some preliminary investigations have shown that addition of traces of acid or alkali do not inhibit the effect of shaking, and on the other hand I found it impossible to reactivate a shaken serum by means of decreasing or increasing its H' -concentration. I intend however to continue my research on these lines, since I can confirm Noguchi's and Bronfenbrenner's observation, that it is possible only by means of adding some alkali to an E-piece, obtained by Liefmann's method, to restore full haemolytic action. But I shall deal with this in a later communication.

As already mentioned, P. Schmidt considers complement to be a ferment, an entity, which is adsorbed on the globulin surfaces in all processes, leading to their precipitation. I readily recognise how easily the main features of the processes involved in the shaking inactivation can be explained by this conception, but there are many points, the explanation of which by this hypothesis seems to be very difficult if not impossible. The following are some of these points :

P. Schmidt's conception does not explain the reason why the albumins in the shaken serum fail to bring the denaturated particles into solution, whereas fresh albumin will do so. Further the reactivation of C_{sh} by C_{th} , the fact that M_{th} has a reactivation effect on C_{sh} , whereas M_n is ineffective, and that such a M_{th} after being heated loses its activity with E_n but not with C_{sh} . The observation that an E_n being submitted to Berkefeld filtration remains active with a C_{sh} , but not longer with a M_n .

Last but not least the observation that a serum, submitted to the effect of dilution and standing, becomes inactive, if rendered isotonic, in spite of the euglobulins being in complete solution, etc.

I claim on the other hand to have shown, that at least as many of the observations can be equally well explained by assuming no substrate for a complement at all, but the necessity of certain physical conditions. I hope later to be able to bring forward more evidence in support of this conception.

GENERAL CONCLUSIONS.

It has been attempted to show that at least most of the experimental results, a summary of which is already given above, can be explained by assuming that shaking produces alterations of the physical conditions in the serum, which by themselves account for the loss of complementing power of the serum, and that the conception of a complement being a ferment, the action of which is due to the presence of a certain substrate, is not necessary. But there are some observations left unexplained by either conception, due to lack of further experimental evidence.

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STUDIES ON THE BERKEFELD-FILTRATION OF COMPLEMENT.

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INTRODUCTORY.

IN view of the divergent results obtained in recent work dealing with the effect of Berkefeld filtration on the haemolytic complement of serum, I think that the publication of the results which I have obtained during some investigations, which I have been carrying out on the inactivation of complement by shaking, may not be without value.

Muir and Browning (1909) found that by filtration through a Berkefeld filter the complement of a guinea-pig serum is retained by the filter to a large extent—sometimes indeed completely,—but if more serum is filtered it passes through. Neufeld and Andrejew (1909) and Andrejew (1910) found that out of all immune bodies in serum, complement is the most completely retained by filtration through Kieselguhr. Andrejew showed further that the relative loss of complement was considerably increased when the serum was previously diluted, no more complement passing through if the serum was diluted 1 : 10. A detailed investigation of the filtrability of complement was then undertaken by P. Schmidt (1911, 1912). He confirmed the observation of Andrejew in regard to the effect of dilution. His explanation was that the colloidal complement ferment, which he considers to be an entity, becomes less dispersed by dilution and therefore more easily retained by the filter. If undiluted, the serum proteins, especially the albumins, have a protecting influence upon the complement, which passes through the filter in the course of prolonged filtration, because the albumins cover the whole Kieselguhr

surface, thus shutting off the adsorbent filter surface from the complement. When he filtered the same serum repeatedly through the same filter, he found a decrease of proteins corresponding with a steadily increasing complementing power of the filtrate.

The results of my first experiments proved to be very contradictory, and not until I had made the technique as uniform as possible did I get uniform results, which could be reproduced under given conditions. There is no doubt that in the filtration of serum an adsorption on the filter material takes place, and it is therefore evident that according to the relations between filter surface and the filtrans different results can be obtained. I think it therefore necessary to have the size of the filter stated as well as the quantity of the filtrans. The fact that these data are wanting in most papers, renders a comparison of the experiments difficult, and on the other hand may explain the divergent results which the literature contains. That *ceteris paribus* also, the velocity of the filtration, dependent on the pressure, the H⁺-concentration, and the presence of neutral salts play an important part, will be shown at the end of this paper.

Technique of experiments.

The Berkefeld candles employed were cylinders of about 40 mm. height and 14 mm. diameter. The filtration was directed from outwards to inwards and was effected by a suction water pump having a pressure of about 680 mm. Hg.

During filtration the whole surface of the filter was kept covered by the liquid. On every occasion the filter was cleaned by filtering a hot diluted solution of NaOH followed by distilled water until the filtrate was neutral to phenolphthalein. In those cases where the filter was not completely dried before use (dist. water or 0.85 % saline remaining in the filter) the first portion of the filtrate was rejected. The complementary function of the serum was tested for its haemolytic power in combination with sensitized sheep red corpuscles, of which a 2.5 % emulsion in 0.85 % saline solution was used. The haemolytic immune-serum was inactivated rabbit serum, the single lytic dose of which was 0.00125 c.c. for 1 c.c. of the red cell emulsion and 0.1 c.c. complement containing serum.

In the haemolytic tests the following schema has been adopted to illustrate different degrees of haemolysis.

-  No haemolysis.
-  Faint trace of haemolysis.
-  Very slight haemolysis.
-  Well-marked haemolysis.
-  Half haemolysed.
-  Strong haemolysis.
-  Very strong haemolysis.
-  Nearly complete haemolysis [a slight trace of unlysed cells at the bottom].
-  Complete haemolysis.

Experimental part.

Exp. 1. Filtration of fresh guinea-pig serum undiluted and 1:10 diluted with 0.85 % saline through a Berkefeld candle (50×14 mm.).

Haemolysis of 0.5 c.c. sensit. red cell emulsion, after 1 hour at 37° and 15 hours at room temperature. AB dose = 0.0025.

		C.c.	1.0	0.5	0.25	0.15	0.0
$\frac{1}{10}$ dil. compl. serum	...	...					
Undiluted through Berkefeld filtered, then 1:10 diluted with 0.85 % saline, one part of filtrate=3 c.c.	{	I					
		II					
		III					
		IV					
$\frac{1}{10}$ with saline diluted through Berkefeld filtered, each part of filtrate =10 c.c.	{	I					
		II					
		III					
		IV					

Exp. 1 shows that the loss of complementing power of undiluted serum seems to be relatively small compared with the nearly total loss in the case of the serum which has been diluted 1:10 with saline. It will be observed however that the absolute amount of native serum which passes the filter after filtration of 40 c.c. of the diluted serum is less than the amount of undiluted serum which has passed through the filter during the filtration of 6 c.c. undiluted serum. This experiment when repeated always gave similar results indicating that the relatively smaller loss of complement in an undiluted serum is only apparent.

If according to P. Schmidt the complement is supposed to be a ferment and an entity, it will be adsorbed on the Kieselguhr surface either

owing to its action on the surface tension or by electrical interaction [anomalous adsorption]. Adsorption is going on so long as the surface is not completely saturated, and saturation is more quickly reached in the case of undiluted serum. In the case of diluted serum much more liquid is required to saturate the adsorbing surfaces, so as to finally allow the complement to pass through. There is therefore no need to assume a lesser degree of dispersity of the complement ferment as an effect of dilution, for which there is also no other evidence. P. Schmidt supposes further that the complement already adsorbed on the filter surface will be freed in the course of the filtration by the protecting effect of the albumins, which accumulate at the filter surface. This seems to be difficult to understand, for there is no evidence that the albumins and the complement have a different action on the surface tension, and it is difficult to see why the albumins are not immediately adsorbed, thus preventing the complement from being adsorbed at all.

On the other hand it may be possible to assume that the complementing power of a serum is not bound on a certain substrate, but requires certain physical conditions of the serum, namely the relative state of the different serum proteins as regards their quantity and their degree of dispersity, which latter is closely connected with the amount of neutral salts present and the H⁺-concentration. If a serum which has complementing power is filtered through a Berkefeld filter, the whole filtering surface must first of all be changed by adsorption of proteins and its adsorptive power completely satisfied, before the serum can pass unchanged as regards its complementing power. This requires in a given filter a certain amount of proteins, and this condition of complete adsorption in the case of an undiluted serum is naturally more quickly reached. The first fraction of the filtrate is very poor in protein. With the increasing protein content the complementary function of the filtrate increases, finally reaching the original value of the serum before filtration. If such a serum is repeatedly filtered through the same filter, as P. Schmidt did 16 times, the protein content was found by him to steadily decrease, whereas the complement activity increased. The following Exp. 2 does not confirm this statement of P. Schmidt. But owing to the absence of detailed data in his paper the experiments can only be compared to a restricted degree.

Exp. 2. 30 c.c. of fresh undiluted guinea-pig serum was 17 times filtered through the same Berkefeld filter.

After each filtration 0.5 c.c. was taken and diluted 1 : 10 with 0.85 % NaCl.

Haemolysis after 1 hour at 37° and 1 hour at room temperature.

0.5 c.c. sensit. red cell emulsion. AB dose = 0.0025 c.c., the total volume being 1.5 c.c.

		1.0	0.5	0.25	0.15
Compl. serum ($\frac{1}{16}$) normal					
Berkefeld filtrate	1				
	2				
	3				
	4				
	5				
	6				
	7				
	8				
	9				
	10				
	11				
	12				
	13				
	⋮				
	17				

Exp. 2 shows that the complementing power of the serum, which is still well marked after the first filtration, is slowly but steadily decreasing with the number of filtrations. After the 12th filtration had been effected, the complementing power was lost and did not reappear on further filtration. The first part of the first filtrate was almost free of protein as in Exp. 1. Thereafter the velocity of adsorption is asymptotically decreasing, *i.e.* so long as filtration is going on there will be adsorption but finally to an indefinitely small extent.

From Exp. 2 either a steadily progressing adsorption of complement ferment may be assumed or the relations of the proteins may be steadily changing till the alteration is such that complementing power is no longer possible.

The saturation of the filter surface can also be effected by the previous filtration of a thermo-inactivated serum as has been done by Muir and Browning (1909). A similar experiment was made by Holderer (1912), who effected the filtration of diastase through a Chamberland candle after the previous filtration of an albumin solution.

In order to decide whether the different proteins in the serum undergo a different adsorption on the Kieselguhr surface of the filter, a series of experiments was made to show how far an inactive Berkefeld filtrate can be reactivated by the addition of the serum fractions which have been obtained by the CO_2 method of Liefmann. For the sake of brevity I call the fractions M- and E-piece respectively, the M-piece containing the euglobulin and a relatively small part of the pseudoglobulin, the E-piece containing all the albumin and the main bulk of the pseudoglobulin.

Browning and Mackie (1912) found that no reactivation either by M-piece or by E-piece takes place, but later (1913) they found a restitution possible but not with regularity. P. Schmidt showed that a filtrate which was ineffective by itself could be reactivated by a small amount of fresh serum which, though inactive by itself, produced reactivation by a summation of effects. In accordance with his own conception that the albumin fraction contains always traces of the complement ferment, the main bulk of which is adsorbed on the surfaces of the euglobulin precipitated by dilution and CO_2 , he concludes that a complete removal of the complement by means of adsorption on the filter is scarcely to be expected. The complement in the inactive filtrate is only in a state of subactivity. He found further that such a filtrate can replace an E-piece, *i.e.* that it can be reactivated by the M-piece.

Exp. 3. Reactivation of the filtrates of Exp. 1 by the fractions of the normal guinea-pig serum (CO_2 method).

Haemolysis after 1 hour at 37° and 18 hours at room temperature of 0.5 c.c. sensit. red cell emulsion.

AB dose = 0.0025 c.c. Total volume = 1.5 c.c.

		(1) filtrate				(1 ₁₀) filtrate				0.85% NaCl		
		I	II	III	IV	I	II	III	IV	M-p.	E-p.	
(1 ₁₀)	0.5 c.c. E-piece											
(1 ₁₀)	0.5 c.c. M-piece											

Exp. 3 shows that in the case of the serum which has been filtered in the undiluted state no reactivation by any fraction can be obtained as long as the filtrate is inactive by itself. The filtrates of the diluted serum however could be reactivated by M-piece in an increasing degree, which means that with the progress of the filtration more albumin

passed through, which then increases the solubility of the M-piece. Such a Berkefeld filtrate acts therefore as an E-piece, just as P. Schmidt found. The addition of E-piece however to a Berkefeld filtrate of a diluted serum is not without effect. Either complement ferment passed through the filter in a state of subactivity, but becoming active by summation with the traces of complement ferment in the E-piece, or on the other hand the filtrate still contains globulins, which together with the globulins of the E-piece give a summation effect by the excess of albumin. In any way the restitution effect of the albumin fraction is very small.

In analogy with the result of Exp. 2, that in repeatedly filtering the same serum through the same filter a slow but steady decrease of complement occurs, the following Exp. 4 shows what happens if the same serum be filtered through a series of different filters.

Exp. 4. 20 c.c. of an undiluted fresh guinea-pig serum was filtered through a series of Berkefeld candles under similar conditions.

After each filtration 2 c.c. of the filtrate was removed, diluted 1 : 10 with 0.85 % saline and their haemolytic power as well as the effect of restitution by E-piece or M-piece investigated.

Control reactions with E-piece and M-piece worked satisfactorily.

Haemolysis of 0.5 c.c. sensit. red cells (AB dose = 0.0025 c.c.) after 1 hour at 37° and 15 hours at room temperature.

	1.0	0.5	0.25	0.0	1.0 c.c. E-piece + 1.0 c.c. filtrate	1.0 c.c. M-piece + 1.0 c.c. filtrate
$\frac{1}{10}$ normal compl. serum						
Filtrate N ₂ O						
I						
II						
III						
IV						

Exp. 4 shows that in this case the complementing power of the serum decreases rapidly with each filtration. The filtrate thus rendered inactive could not be reactivated by any means.

Filtration of the CO₂ fractions.

In connection with these experiments I undertook the isolated filtration of the CO₂ fractions and give in the following a record of some typical experiments.

Filtration of Complement

Filtration of E-piece.

Exp. 5. To decreasing amounts of M-piece diluted $\frac{1}{10}$ with 0.85 % saline 0.5 c.c. of filtered E-piece diluted $\frac{1}{10}$ is added.

Haemolysis after 1 hour at 37° and 1 hour at room temperature of 1 c.c. 2.5 % sensit. red cell emulsion. AB doses = 0.0025 c.c., the total volume in each tube being 2 c.c.

(1/10) M-piece, c.c.		0.5	0.25	0.15	0.1	0.0
+ 0.5 (1/10) E-piece		...	■	■	●	●
E-piece rendered iso-tonic after filtration	I	□				□
	II	□				
	III	□				
E-piece rendered iso-tonic before filtration	I	□	□	□	□	□
	II	■	■	■	■	□
	III	■	■	■	■	□

Exp. 5 shows first of all the fundamental difference in the filtrability of an E-piece whether rendered isotonic or not. The passage of the E-piece was impossible before the addition of salt. The salted E-piece behaved similarly to a complement serum in so far as filtration is concerned, that is, the longer the filtration the more it comes through.

Muir and Browning have already found that the permeability of the Berkefeld filter is increased by the addition of salt to the serum, thus rendering the serum hypertonic. The serum is then brought back to the isotonic condition by the dilution with water. This has been later confirmed by Manol and Nowaczynski (1910). The last mentioned authors succeeded in regaining the complement retained in the filter by filtering immediately afterwards a hypertonic solution of NaCl. Muir and Browning did not succeed in this experiment. It must however be mentioned that Manol and Nowaczynski worked with the Chamberland filter.

Before attempting to give an explanation of the part played by the presence of salts, I will refer to another experiment, which shows this phenomenon.

Exp. 6. Effect of Berkefeld filtration upon complement serum diluted 1 : 10 with distilled water.

Haemolysis of 0.5 c.c. sensit. red cell emulsion (AB dose = 0.0025 c.c.) after 1 hour at 37° and 15 hours at room temperature.

	C.c.	1.0	0.5	0.25	0.15
Guinea-pig serum after standing 24 hours, diluted $\frac{1}{10}$ with 0.85 % NaCl					
The same serum but diluted $\frac{1}{10}$ with aqu. dist. After 4 hours standing at room temperature, there was very marked cloudiness, which completely disappeared by rendering the serum isotonic					
The serum thus treated and rendered isotonic + 1.0 c.c. ($\frac{1}{10}$) E-piece					
The serum thus treated and rendered isotonic + 1.0 c.c. $\frac{1}{10}$ M-piece					
After 4 hours standing at room temperature and before being rendered isotonic, the serum was filtered through a Berkefeld candle and the clear-looking filtrate rendered isotonic					
The isotonic Berkefeld filtrate + 1.0 c.c. ($\frac{1}{10}$) E-piece...	...				
The isotonic Berkefeld filtrate + 1.0 c.c. ($\frac{1}{10}$) M-piece...	...				

Exp. 6 shows first of all the influence of the dilution with aqu. dist. upon a complement containing serum. As I have pointed out in another paper (1914) the inactivation phenomenon of Sachs and Teruuchi (1907) by 1 : 10 dilution with aqu. dist., and standing a certain time, occurs only in absolutely fresh serum. The serum in Exp. 6 was 24 hours old and was not yet inactivated after 4 hours standing at room temperature when diluted 1 : 10 with aqu. dist. The complementing power however was lessened. The complete restitution by E-piece and the absence of such an effect by M-piece are in favour of some alteration of the albumins. If the diluted and cloudy serum after 4 hours standing, and before being rendered isotonic, was filtered through a Berkefeld filter, the serum became clear, just as if it had previously been salted, but if the filtrate was rendered isotonic it proved to be inactive and incapable of being reactivated by E-piece. The filtrate however still contained traces of euglobulin because the passing of CO₂ through it gave rise to a slight opalescence. Only if the serum stood for many days diluted 1 : 10 with aqu. dist. did I succeed in completely filtering off the euglobulin. As in Exp. 5 the absence of a sufficient amount of salts inhibited the passage of the albumins, for the restitution by the addition of fresh M-piece to the filtrate proved to be very small, but still better marked than the effect of E-piece.

A comparison of the different effects of filtration through paper or through a Berkefeld filter upon the action of E-piece is given in the following Exp. 7.

Effect of filtration upon E-piece.

Exp. 7. Guinea-pig serum after standing 24 hours in the cool room was diluted 1 : 8 with aqu. dist. and made acid by CO₂.

The precipitate was once washed and immediately before use dissolved in 0.85 % NaCl in 1 : 20.

The supernatant fluid (E-piece) was treated in the following way :

I. The supernatant fluid was rendered isotonic and remained otherwise untreated.

II. The supernatant fluid was filtered through soft filter paper and then rendered isotonic.

III. The supernatant fluid was filtered through hardened paper and then rendered isotonic.

IV. The supernatant fluid was rendered isotonic and then filtered through a Berkefeld candle.

Haemolysis of 0.5 c.c. sensit. red cell emulsion after 1 hour at 37° and 15 hours at room temperature. AB dose = 0.005 c.c., the total volume in each tube being 2.5 c.c.

$\frac{1}{20}$ M-piece	1.0	1.0	1.0	1.0	1.0	1.0	
NaCl sol.	0.0	0.5	0.75	0.85	0.9	1.0	Half saturation with ammonium sulphate
C.c.	1.0	0.5	0.25	0.15	0.1	0.0	
Of: I							Heavy precipitate.
II							" "
III							" "
IV							Only a slight dimness occurs, the liquid being almost clear.

Exp. 7 shows that a Berkefeld filtered E-piece is rendered incapable of giving an haemolytic effect with M-piece. The filtration removes about all the pseudoglobulins, which the albumin fraction contains, while the filtration through paper does not affect the pseudoglobulins. This is demonstrated by the effect of half saturation with sulphate of ammonium. In connection with this it is of interest to note that Browning and Mackie (1914) found recently that the pseudoglobulin represents the main factor as far as the complementing haemolytic power of a serum is concerned.

In regard to the filtration of the isolated M-piece, Exp. 6 shows that already without the presence of sufficient salts the euglobulins, being in suspension, are retained in the filter. Only a very minute portion passes with the diluted serum, probably owing to the small

amount of salts and to the protecting influence of the serum albumins. The filtration of the M-piece diluted 1 : 10 in 0.85 % saline has an effect similar to that of the filtration of the salted E-piece.

Exp. 8. M-piece obtained by the CO₂ method from a fresh guinea-pig serum was dissolved by 0.85 % NaCl solution in 1 : 10, and then filtered through a Berkefeld filter.

Each fraction of the filtration was of a volume of 10 c.c.

Haemolysis of 0.5 c.c. filtrate + 0.5 c.c. $\frac{1}{10}$ E-piece + 0.5 c.c. sensit. red cell emulsion (AB dose = 0.005 c.c.) after 1 hour at 37° and 15 hours at room temperature.

$\frac{1}{10}$ M-piece Berkefeld filtrate	I	II	III	IV	V	VI	VII	VIII
+ 0.5 c.c. E-piece $\frac{1}{10}$...								

Exp. 8 shows that the more prolonged the filtration the more M-piece passes through the filter. It behaves therefore just as the salted E-piece or the whole serum under similar conditions.

The effect of filtration upon the isolated fractions however must not be taken to represent what occurs if the two mixed together are filtered as in the case of a serum. Apart from other factors the different influence upon the surface tension of the medium by the different proteins would be already sufficient to vary the effect of filtration.

Theoretical part.

In attempting to give an explanation of all the phenomena observed in the Berkefeld filtration of serum it must be remembered that the filtration consists of several different processes, namely :

1. The mechanical retention of particles.
2. The adsorption on the filter material, in so far as surface tension is concerned.
3. The phenomena caused by the movement of the liquid in the capillaries of the filter causing either anomalous adsorption or coagulation.

The mechanical retention of particles occurred in the above described experiments only when the euglobulin was in suspension. It is however easy to demonstrate by means of an ultramicroscope the presence of many particles in the filtrate which escaped retention, but in view of the relatively large size of the Berkefeld filter pores (J. A. Craw, 1908) this is to be expected. It is also possible that in using a dry filter

the rushing of the liquid in the capillaries is so great that suspended particles may be carried through capillaries which otherwise would hold them back. This is shown by Grenet (1910) to occur in the case of filtration of bacteria. On the other hand, some of these small particles may consist of the filter material which appear especially when new filters are employed.

A more important effect upon filtration of serum is exerted by the adsorption of the Kieselguhr surface of the filter. According to Gibbs' thermodynamic principle those substances in the serum which lower the surface tension are adsorbed. These latter are principally the serum proteins, the albumins and the globulins. F. Bottazzi (1912), M. J. Gramenitzky (1913) and other authors have shown that the serum albumins as well as the serum globulins lower the surface tension of water, but the latter in a lesser degree. This may be the reason for the protective influence which the albumins have upon the stability of the dispersity of the globulins. I give below some data showing the influence of the Berkefeld filtration upon the surface tension of the filtered liquid. The figures represent the number of drops given by a stalagmometer of Traube, and calculated as mentioned in my former paper (*this Journal*, 1913, p. 316).

Table showing influence of Berkefeld filtration upon S.T.

Dist. water	...	...	...	...	...	...	100.0
M-piece diluted 1 : 10 with 0.85 % NaCl	...	...	...	...	...	...	102.16
"	"	"	"	filtered through Berkefeld filter			100.17
E-piece				Untreated		Filtered through Berkefeld	
diluted 1 : 4, not isotonic	...	...	...	104.30		102.41	
" 1 : 8, "	...	...	...	102.77		100.76	
" 1 : 10, isotonic	...	...	...	104.95		102.51	
Serum diluted 1 : 10 with				0.85 % NaCl		Aqu. dist.	
" untreated	...	...	...	103.67		101.57	
" heated $\frac{1}{2}$ hour at 56° C.	...	...	...	106.87		106.38	
" filtered through Berkefeld filter	...	...	...	103.06		100.56	

The table shows clearly that substances which lower the surface tension are kept back by filtration, thus producing a higher surface tension of the filtrate.

There are however other substances in the serum which lower the surface tension. Apart from the haemoglobin which according to Iscovesco (1911) lowers the surface tension of water, but the presence of which in the serum can be avoided, there are the pigments and the

lipoids of the serum as well as salts of fatty and gallic acids (J. Traube, 1908). The usual method of preparing a serum albumin by means of salting out with the sulphate of ammonium involves the pigments and the lipoids, the pigments coming down mainly with the albumin. It is therefore a matter of doubt whether the relatively large decrease of surface tension produced by the serum albumins is not at least partly due to the accidental presence of these substances, the more so as Iscovesco (1910) found that pure egg albumin even increases the surface tension of water. It would be of interest if egg- and serum-albumin are really different in their effect upon surface tension. That these serum pigments are readily adsorbed follows from the observation that the first parts of the filtrate are colourless; the serum colour reappears with further filtration.

As far as my experience is concerned, I agree with the statement of Bottazzi, that the serum albumins lower the surface tension of water (H. Schmidt, 1914). The albumins therefore tend to become adsorbed. If the serum has previously been heated, it has been shown by Traube, M. J. Gramenitzky, L. Berczeller, H. Schmidt and others, that a well-marked decrease of surface tension occurs. According to L. Berczeller (1913) this loss of surface tension is due to the production of albumoses and peptone.

From these observations it is easy to understand that a preliminary filtration of a thermo-inactivated serum (Muir and Browning) or of peptone (Holderer, 1912) facilitates the filtration of serum. Adsorption of the albumins diminishes their protective influence upon the dispersity and the solubility of the euglobulins. The dispersity especially of the euglobulins is decreased and as a consequence their filtrability is also decreased. But this may not be the only cause of the retention of the euglobulins. According to recent work of H. Chick (1913) the globulins form soluble compounds with the salt (NaCl) which are electrically neutral. As there is adsorption of the ions of the dissociated salts in the serum during filtration, as will be explained later, the globulin salt compounds tend to dissociate and become electrically charged, which renders them liable to anomalous adsorption. Quantitatively the amount of euglobulin in the serum is comparatively very small, and therefore its partial removal alters the mass relations of the proteins far more than a removal of a corresponding part of the albumins or the pseudoglobulins. This may help to explain the E-piece action of a Berkefeld filtrate.

In order to explain the influence of salts upon filtration through

a Berkefeld filter I would like to draw attention to a phenomenon, first described many years ago, but so far as I am aware not yet taken into account in the filtration of serum.

Quincke (1861) observed that in passing a liquid through a capillary tube a potential difference occurs between the capillary wall and the liquid. Helmholtz (1879) found an expression for the electromotive force thus developed according to which it is, apart from other relations, directly proportional to the hydrostatic pressure and inversely proportional to the viscosity and the electric conductibility of the liquid in question.

This has been confirmed later by J. Perrin (1904, 1905, 1906). Fr. Fichter (1911) and N. Sahlbohm (1910) drew attention to this phenomenon in explaining the precipitation which colloids with electro-positively charged particles undergo, when passing through capillaries, the diameter of which does not exceed 0.15 mm. N. Sahlbohm demonstrated this phenomenon to be a consequence of the movement of the liquid through the capillary and to be independent of the nature of the material of the capillary walls. Where there is no movement only adsorption takes place. The movement of the liquid produces a negative charge of the wall against the water, while in the case of electro-positively charged colloids, the colloidal particles are positively charged against the water as their dispersion medium. If the potential difference thus produced be large enough to overcome the positive charge of the particles the latter lose their charge and owing to surface tension aggregate and precipitate.

Now the unsalted E-piece obtained by dilution with water and acidifying with CO_2 has a distinct acid reaction and the protein particles in it are therefore positively charged (W. Pauli). It seems to me very probable that such an E-piece being an electro-positive colloid is to a large extent precipitated in the small capillaries of the filter and therefore not able to pass through a Berkefeld filter (cf. Exp. 5).

On the other hand, it is possible to assume that negatively charged protein particles will show a tendency to be repulsed by the negative charge of the Kieselguhr surface. This occurrence would inhibit the adsorption process and facilitate the passage. Positively charged particles would however show a tendency to adsorption, and the greater this potential difference the greater would be this tendency, but in the course of adsorption the potential difference is decreasing owing to the neutralising effect of the adsorbed particles and with that the adsorption decreases as well, till finally no more adsorption takes place.

The presence of electrolytes such as NaCl influences the phenomenon in the following manner: the NaCl is dissociated; its kation tends to neutralise the electric charge of the filter surface. This causes a decrease of the potential difference and therefore less repulsion of negative particles and less attraction or adsorption of positive particles. The influence of NaCl consists therefore in facilitating the adsorption of negatively charged particles and in diminishing the adsorption of positive particles. This is in analogy with the anomalous adsorption of dyes on filter paper described by W. M. Bayliss (1906) and may explain the results of Exp. 5.

It must however also be remembered that the addition of salts decreases a little the viscosity of proteins (W. Pauli), and increases the electric conductivity to a large extent. This latter factor will therefore, according to Helmholtz's formula, diminish the electromotive force produced by the movement in the capillaries, and in this way salts tend to prevent the precipitation of acid proteins. In addition to this the presence of salts (NaCl) decreases the degree of acidity and therefore the positive charge of the particles in an acid protein solution (H. Chick and C. J. Martin, 1913).

Finally I would like to draw attention to some experiments made by Holderer because these experiments, in spite of being made with Chamberland candles, verify to some extent the above described conceptions.

M. Holderer (1909, 1910) and M. Holderer and G. Bertrand (1910) found that many ferments which do not pass a Chamberland filter if in a medium neutral to methylorange ($H^+ = 10^{-4.4}$) will promptly do so if the acidity is reduced to the neutral point of phenolphthalein ($H^+ = 10^{-8.3}$). By the addition of neutral salts however Holderer (1910) succeeded in filtering the ferments even if the medium was only neutral to methylorange.

SUMMARY.

In the Berkefeld filtration of a fresh guinea-pig serum, adsorption on the filter surface takes place, and the serum passes unaltered, if the saturation of the adsorbing surface has become complete.

This adsorption involves most probably first the albumins, which process then secondly influences the filtrability of the globulins. It is also shown that the physical conditions of the serum as regards salt concentration and dilution as well as H^+ -concentration modify the effect of filtration to a large extent.

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THE IDENTIFICATION OF THE PATHOGENIC MEMBERS OF THE TYPHOID-COLON GROUP OF BACILLI.

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EVERY year an immense amount of work is done in this country on the isolation and examination of organisms belonging to the typhoid-colon group of bacilli. Much of it is concerned with the routine examination of water, but a considerable proportion is directly concerned with the examination of excreta, and in many laboratories an extensive experience has been gained of satisfactory methods of isolating and identifying the important pathogenic members of the group. This year, however, war conditions have brought about an enormous expansion in the amount of such work, and at the same time exceptional urgency for its rapid execution. Partly as a result of this, there are already engaged in the examination of excreta many workers whose previous bacteriological experience has been mainly concerned with other departments, and who have not hitherto had to deal with the routine examination of large quantities of such material; and their number is likely to increase still further, as we must anticipate a large and progressive increase in the volume of work with the arrival and continuance of the hot weather. Such men have not the time to look up the recorded experience of others, and perhaps not always the opportunity to consult those whose previous work has made them more familiar with the best methods to adopt. It is hoped that the present paper may be of service to them, based as it is not only on the literature but also on the very large experience of this Institute for many years past, both in the identification of the pathogenic organisms and in the rapid examination of large quantities of material.

The typhoid-colon bacilli are all Gram-negative rods which grow freely on ordinary media at 20°–37° C. and do not form spores, and they are subdivided into two main groups, according to their action on lactose. Those which ferment lactose, producing from it acid with or without

formation of gas, are labelled with the general name of *B. coli*, and do not concern us further here. They are practically ubiquitous, and many of them are pathogenic in certain regions or under certain conditions, but they do not produce epidemic disease with predominantly intestinal symptoms. The lactose non-fermenters are again divisible into two main groups by their action on gelatin. The gelatin liquefiers pass into the great group of *B. proteus* (some of them, like *B. coli*, occasionally pathogenic), and with these also we are not further concerned. All the organisms of the group recognized as regularly producing epidemic intestinal disease are included amongst the lactose non-fermenters and gelatin non-liquefiers.

The application of these two tests excludes the great majority of the organisms which belong to the colony-typhoid group, and the first elimination is largely effected by the addition of lactose and a suitable indicator to the medium on which the material is plated in the first instance. Thus in MacConkey's medium any organism which has produced acid from the lactose affects the neutral red in the medium, and the colonies are red, lactose non-fermenters remaining colourless. But there is a source of fallacy in the primary subdivision which must be continually kept in mind. There is a fairly extensive group of organisms which are really akin to the lactose fermenters but do not ferment lactose in the first few days of growth. After an interval of perhaps as long as six to seven days' growth on the sugar they produce descendants which do ferment it, but in the first period they are lactose non-fermenters. In practice one must include in the first selection these late lactose fermenters along with the non-fermenters. Before the final diagnosis is made they will have declared themselves, and if it is realized that the first selection has not been absolutely exclusive of all *B. coli*, they rarely cause serious trouble in the subsequent procedure.

This difficulty of variable characteristics is one which runs through the whole of bacteriological classification. The classification is based on certain characters, which in this group consist of behaviour towards certain substances (carbohydrates, gelatin, milk, etc.) and of serological reactions. Probably none of the characters of any particular organism is so absolutely fixed that it cannot possibly be altered. The difficulty, however, is, so far at least as is known at present, only occasionally a practical one. It may be and is possible under certain conditions to induce a typhoid bacillus to ferment lactose, or to breed a Gaertner bacillus which does not produce gas from glucose; but in clinical typhoid or Gaertner infections the one is not in fact a lactose fermenter, and the other produces gas, and in the practical conditions of the present time any organism which does not conform to the characters described may be rejected as not one of the known pathogenic members of the group. (The only exception which is perhaps worth mention here is in the case of *B. paratyphosus* A. This organism normally produces gas

from the carbohydrates it ferments, but occasionally the production of gas is very slight or absent on some or all of them. Suspicion will be aroused by the fact that acid is produced only in the sugars attacked by the typical bacillus, especially if gas is present in one or more of them, and an agglutination test will settle the point.)

The genuine lactose non-fermenters and gelatin non-liquefiers, then, are the important group, and of these a table is given. This explains itself. No attempt has been made to make it exhaustive or strictly systematic. It is intended only as a guide in practical work, and is in fact constructed in accordance with the steps recommended for actual procedure later on; but it contains all the diagnostic characters required for the recognition of the pathogenic bacteria, whatever the system of classification adopted. In two instances, namely, the mannite fermenting group of dysentery bacilli and *B. paratyphosus* B. further subdivisions are frequently recommended. These have not been included in the table, because no result of immediate practical importance is gained thereby, but they require some notice.

Mannite Fermenting Dysentery Bacilli.—This is a group where classification presents exceptional difficulties. In the case of infection with typhoid, Gaertner, or the other pathogenic bacilli of the typhoid-colon type, we have to deal with organisms which present a fairly definite and uniform combination of characters. But in the dysentery due to mannite-fermenters such uniformity does not exist. They are all non-motile and all produce acid on mannite, and of course they do not ferment lactose or liquefy gelatin, but, beyond this, uniformity either of cultural or serological characters ceases. In different epidemics the causal organisms may conform completely to a recognized type, such as Flexner's original bacillus, or they may differ in one or more particulars from any of the types previously established. Under such circumstances detailed subclassification is of no value for our purpose here. If a table were constructed subdividing into groups by the successive application of different tests, it would have a certain value in systematic differentiation, and the separation of a subgroup, that of *B. dysenteriae* Y. by the use of maltose has become in some sort classical. But even the academic value of such differentiation is doubtful because of the uncertainty whether these organisms are stable in the characters they are found at one time to possess or may not change in one character or another from time to time. The system adopted in the table is to regard all organisms which are agglutinated by Y serum as possible causes of dysentery. *B. dysenteriae* Y is an organism very closely related to Flexner's bacillus, from which it differs in the fact that it does not ferment maltose at the time of isolation, and in its variable production of indol. Serum prepared with it is found to agglutinate all the tested strains of clinically pathogenic bacilli except the bacillus of Strong. It also agglutinates a number of organisms agreeing with the pathogenic

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TABLE I.

Non-Fermenting. GELATIN.		Liquefying. <i>B. proteus</i> Group.		No Change. (Pathogenic: <i>B. dysenteriae</i> Shiga, Morgan No. 1.)		(Pathogenic)	
				GLUCOSE.			
No Change. None pathogenic. <i>B. alcaligenes</i> Group.		Acid Only. SHIGA SERUM.		Acid and Gas. Only pathogenic or- ganism is Morgan No. 1. Other Characters:		Motile (Typhoid). TYPHOID SERUM.	
Positive. <i>B. dysenteriae</i> Shiga.		Negative. None patho- genic.		1. Ferments no other carbo- hydrates (ex- cept galactose and levulose).		Positive. <i>B. typhosus</i> .	
Other Characters:				2. Produces indol.		Other Characters:	
1. Non-motile.				3. Acid, followed by alkali, in milk.		1. Permanent acid in milk.	
2. Acid, followed by alkali in milk.				4. Motile.		2. No indol.	
3. No change in saccharose, dul- cite (or sorbite).						3. No change in saccharose.	
4. No indol.						4. Acid in glucose (and sorbite).	
						Other Ch	
						Flexn	
						1. Acid	
						in m	
						2. No c	
						dul	
						3. Prod	
						4. Acid	
						[<i>B. dys</i>	
						ferme	
						prod	

	LACTOSE.	GELATIN.	MANNITE.	GLUCOSE.	SACCHAROSE.	DULCITE.	MALTOS.	SORBIT.	SALICIN.
<i>B. dysenteriae</i> Shiga ...	0	0	0	A	0	0	0	0	0
Morgan's bacillus, No. 1...	0	0	0	AG	0	0	0	0	0
<i>B. typhosus</i> ...	0	0	A	A	0	0 or late A	A	A	0
<i>B. dysenteriae</i> Flexner ...	0	0	A	A	0	0	A	0	0
<i>B. dysenteriae</i> Y ...	0	0	A	A	0	0	0	0	0
<i>B. dysenteriae</i> Strong* ...	0	0	A	A	A	A	A	A	0
<i>B. paratyphosus</i> A ...	0	0	AG	AG	0	AG	AG	AG	0
<i>B. paratyphosus</i> B ..	0	0	AG	AG	0	AG	AG	AG	0
<i>B. enteritidis</i> Gaertner...	0	0	AG	AG	0	AG	AG	AG	0

A = Acid only; AG = acid and

* There is some uncertainty as to the characters of *B. dysente*

BACILLI.

Fermenting.
B. coli Group.

pe of dysentery.)

Non-motile
 (Stry Group).
 SERUM.

Negative.
 Only pathogenic
 organism is
B. dysenteriae
 Strong (vide text).
Other Characters:
 1. Acid in sacchar-
 ose, glucose, dul-
 cite and sorbite).
 2. Produces indol.
 3. Acid, followed
 by late clot, in
 milk.

Acid and Gas.
 (Pathogenic: Paratyphoid-Gaertner.)

CANE-SUGAR.

No Change. Acid, or Acid and Gas.
 DULCITE. None pathogenic.

Acid and Gas. Acid, or No Change,
 SALICIN. None pathogenic.

No Change. Acid, or Acid and Gas.
 AGGLUTINATION. None pathogenic.

Paratyphoid A
 Serum.
B. paratyphosus A.
Other Characters:
 1. No indol.
 2. Motile.
 3. Acid permanently
 in milk.

Paratyphoid B
 Serum.
B. paratyphosus B.
Other Characters:
 1. No indol.
 2. Motile.
 3. Acid, followed by
 alkali, in milk.

Gaertner Serum.
 Gaertner's bacillus.
Other Characters:
 1. No indol.
 2. Motile.
 3. Acid, followed
 by alkali, in
 milk.

MOTILITY.	LEVULOSE.	GALACTOSE.	DEXTIN.	INULIN.	ADONITE.	ARABINOSE.	RAFFINOSE.	ERYTHRIT.	AMYGDALIN.	ISODULCITE.	GLYCERIN.
0	A	A	0 or Sl. A	0		0	0	Sl. A			
+	AG	AG	0	0		0	0	0	0		
+	A	A	A	0	0	0	A	0	0	0 or A	A
0	A	A	A	0	0	A	A	0	0	0	0
0	A	A	A	0	0	A	A	0	0	0	0
0	A	A	0	0	0	A	A	0	0	A	0
+	AG	AG		0		AG	0		0		0
+	AG	AG		0		AG	0		0		0
+	AG	AG		0		AG	0		0		0

; C = clot; Alk = Alkali.
 ulated are as given by Morgan, *Journ. of Hyg.*, 1911, vol. xi, p. 1.

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or batch of samples on the day succeeding their receipt, it greatly lightens the subsequent labour and clears the way for the investigation of subsequent samples. In circumstances of great urgency or pressure full examination may not be possible. An abbreviated technique must then be adopted, but this involves missing a number of cases discoverable by the fuller method. Such abbreviated methods are given below.

1. Plate the suspected material on MacConkey's medium (bile-salt neutral-red lactose agar), and incubate at 37° for at least eighteen hours. If no colourless colonies are present by that time, replace in incubator for twenty-four further hours and examine again.

2. Colonies must be selected from uncrowded parts of the plates. It is best, until experience has been gained, to select colonies differing in appearance. Pick off five colourless colonies and emulsify each in a few drops of sterile broth. If time allows subsequent inoculation of the sugars, etc., the same day, incubate the broth emulsions for two to three hours before proceeding; if not, proceed at once to inoculate from each emulsion a set of eight tubes, containing mannite, glucose, saccharose, dulcitate, broth, litmus milk, gelatin, lactose. Grow the gelatin at room temperature, the others at 37°.

3. After twenty-four hours proceed to provisional diagnosis. If all colonies have given identical results, it is unnecessary to examine further more than two; if the results differ, specimens of each kind must be considered.

PROVISIONAL DIAGNOSIS.

Reject all organisms fermenting lactose or liquefying gelatin. The remainder fall into three groups according to their action on mannite.

A. *No Change on Mannite.*

The only important organism here is *B. dysenteriae* Shiga, and its identification may be completed almost at once. Morgan's No. 1, an organism frequently associated with epidemic summer diarrhoea, should also be looked for.

No Change on Glucose excludes Shiga; such an organism is probably *B. alcaligenes* (and may be definitely diagnosed if the milk is already alkaline, and no change has occurred in the saccharose and dulcitate tubes).

Acid on Glucose without Gas: makes Shiga probable, practically the only other possibilities being some members of the *alcaligenes* group and some water bacilli. Reject any organisms which have fermented saccharose or dulcitate, and test the others with Shiga serum. Negative result excludes, positive result identifies, Shiga; confirm by absence of motility on the broth tube.

Acid and Gas on Glucose: without change on saccharose or dulcitate makes Morgan's No. 1 probable; confirm by presence of motility.

There remains for subsequent examination in this section only the confirmation of organisms identified provisionally as Shiga.

B. *Acid only on Mannite.*

The important organisms here are *B. typhosus*, the group of dysentery bacilli of Flexner type, and *B. dysenteriae* Strong. Inoculate liberally from the broth tube a second broth tube, and incubate for four to five hours, and test for motility. Meanwhile test with typhoid serum all strains except those fermenting saccharose. Positive result identifies; confirm the same day by presence of motility. Test with Y serum those which ferment cane sugar as well as those negative to typhoid serum. Positive result identifies as a member of the Flexner group; confirm the same day by absence of motility.

Amongst the remaining organisms—that is, those negative to both Y and typhoid serum—there may be: (1) Inagglutinable typhoid and dysentery strains; (2) *B. dysenteriae* Strong. These cannot be recognized without further time, and each organism is to be subcultured daily on broth meanwhile.

For subsequent examination in this section there are thus: (1) Confirmation of identified strains; (2) the unagglutinated strains.

C. *Acid and Gas on Mannite.*

The important organisms are those of the paratyphoid-Gaertner group. Reject at once all which have fermented saccharose or clotted milk. Omit for the present (but do not yet discard) all which have failed to produce acid and gas on dulcete. With the remainder proceed as follows: Inoculate a salicin tube and add it to the set of eight tubes. Then test each organism with three serums in this order: Paratyphoid B, paratyphoid A, and Gaertner. Positive result identifies. Subculture the negative strains daily.

For subsequent examination in this section there remains: (1) Confirmation of identified strains; (2) consideration of unagglutinated strains and those which had not fermented dulcete.

By this preliminary examination we have recognized within thirty hours of isolation the great majority of the pathogenic bacilli of the group and got rid of a very large number of organisms certainly not pathogenic. The remaining unidentified bacilli may include *B. dysenteriae* Strong, or inagglutinable strains of the other pathogenic organisms. Temporary inagglutinability is a phenomenon which while it occurs in only a small proportion of the cases is yet so frequent in the examination of large numbers that we must be prepared to meet it. It usually disappears on repeated subculture on artificial media.

The subsequent examination must be delayed till the result of the indol and milk tests can be obtained. The interval should be six further days—that is, a week from first inoculation of the sugars, etc. It must be always with verification of the lactose and gelatin tests.

SUBSEQUENT EXAMINATION.

A. *No Change on Mannite.*

We have here only to confirm the diagnosis of the organisms identified as Shiga and Morgan. *B. dysenteriae* Shiga does not produce indol, produces no change in mannite, saccharose, or dulcitate, and initial acidity in milk is replaced by alkali; it is non-motile. Morgan's No. 1 produces indol, acid followed by alkali in milk, acid and gas on glucose, no change in other carbohydrates (except levulose and galactose), and is motile.

B. *Acid only on Mannite.**Confirmation of Previous Diagnosis.*

1. *Typhoid Bacillus*.—Acid only and permanently in milk, no indol, no action on saccharose, acid only on mannite and glucose, no change on dulcitate or late acidity, motile. (Non-motile strains occur, but are extremely rare.)

2. *Dysentery of Flexner Type*.—Test for agglutination with Flexner serum. Positive result places the organism among the close allies of Flexner. Typical Flexner produces indol, acid followed by alkali on milk, acid only on glucose and mannite, no change in saccharose or dulcitate, and is non-motile. For further subdivision, *vide supra*.

Examination of Unclassified Strains.

1. *Motile Strains*.—These cannot be dysentery; the only possibility is inagglutinable typhoid. Reject any organism whose characters do not agree completely with those just given for typhoid. Test the remainder with the last subculture on broth and typhoid serum. Positive result identifies definitely; but a negative result with an organism of correct cultural characters must be returned as inagglutinable typhoid. (Preserve such strains for later examination.)

2. *Non-motile Strains*.—Examine the last subculture with Y serum. Negative result leaves the possibility of Strong's bacillus (and non-motile typhoid; note characters). Reject those which have not produced acid only on saccharose. *B. dysenteriae* Strong produces indol, acid on glucose, saccharose and mannite, and should give late clot on milk (*vide supra*).

C. *Acid and Gas on Mannite.*

Confirmation of Identified Strains.—All these produce acid and gas on glucose, mannite and dulcitate, no change on salicin and saccharose, fail to produce indol, and are motile. Paratyphoid A produces acid only and permanently in milk; Paratyphoid B and Gaertner produce acid followed by alkali in milk.

Unidentified Strains.—Reject all which have failed to produce acid and gas on dulcitate, and all which do not conform exactly to the above cultural characters of Paratyphoid A or Paratyphoid B. Test with Paratyphoid A serum the last subcultures of those agreeing with Paratyphoid A; and with Paratyphoid B and Gaertner serum those agreeing with Paratyphoid B. If still negative they are not to be identified as pathogenic, but the strains should be preserved. (*Vide supra* for subdivision of the Paratyphoid B organisms.)

ABBREVIATED METHODS.

These all fail to detect pathogenic organisms in some cases in which the fuller method would have been successful, and the proportion of such failures is greater the more rapid the method. All will detect the greater number, and in periods of stress it is better to examine by a short method in order to cover a larger number of samples. In all of them the first step is to plate on MacConkey's medium and incubate as before for at least eighteen hours.

I.

Pick off five (or more) colourless colonies direct into a mannite tube and incubate till next day. If the result is no change, or acid only, in all tubes, select one only and proceed as follows, according to the action on mannite; if the results differ, take one of each kind:

- (a) *No change.* Inoculate a broth tube, and in four to five hours test for motility. If non-motile, test with Shiga serum; positive result identifies.
- (b) *Acid only.* Inoculate a broth tube, and in four to five hours test for motility. If motile, test with typhoid serum; if non-motile, with Y serum. Positive result identifies as typhoid or mannite fermenting dysentery.
- (c) *Acid and gas.* So many non-pathogenic organisms have this action that it is advisable to inoculate all five organisms into saccharose, dulcite, litmus milk, broth, and gelatin. Next day reject the organisms which ferment saccharose and do not ferment dulcite; and test the others with paratyphoid B, paratyphoid A, and Gaertner serum.

Confirmation of identified strains may be carried out. This method will detect a high proportion of the pathogenic organisms. It misses all inagglutinable strains, and by assuming under (a) and (b) identity of all the five organisms from their action on one carbohydrate, misses a further proportion, and it delays the identification of the acid and gas producers for a further twenty-four hours. It saves the considerable expense of the carbohydrate examination of the (a) and (b) groups, and the labour thereby involved, though more agglutination tests are necessary.

II.

Inoculate five or more colourless colonies into broth, and examine for motility in four to five hours. If motile, test with typhoid, Paratyphoid B, Paratyphoid A, and Gaertner serums. Clinical information may help to determine the order of testing. If non-motile, test with Shiga and Y serums. This is probably the best method when the full examination is impossible. It omits more inagglutinable strains than the last, but is rapid, economical, and will detect most of the pathogenic organisms present.

III.

Test by agglutination with the six serums colourless colonies taken straight from the plate, guiding the order by

clinical information. Examination of the preparation directly it is made will frequently show the presence of motility, and save the labour of examining with the dysentery serums, but the absence of motility under these conditions does not necessarily mean that the organism is non-motile. This method of examination is the most rapid of all, but the proportion of inagglutinable strains missed is still higher under these conditions.

Various modifications of these methods are, of course, possible, but the full method is recommended wherever practicable, and the second abbreviated method as an alternative. There are several ways in which the experienced can save time and increase the probability of finding any pathogenic organisms present. Most important of these is the initial selection of likely colonies, and the appearance of the plate is also of great assistance. The use of large plates or substitutes, on each of which two or more samples can be plated at the same time without mixing, saves a great amount of sterilizing, washing, pouring, etc. The use of plates already warmed on inoculation increases markedly the size of the colonies at the time of examination, and enrichment media for preliminary incubation may increase the chance of detecting the organisms sought. But modifications of this kind cannot be discussed in this paper, and must be left to the individual himself to work out.

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XII. RESEARCHES ON THE INHIBITION PRODUCED BY CERTAIN SERA ON THE COAGULATING POWER OF RENNET.

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(Received February 15th, 1915.)

INTRODUCTION.

Among the numerous papers of the last 20 years dealing with the reaction of rennet and its antibodies, those of Morgenroth [1899], Korschun [1902] and Hedin [1906, 1909] have been of particular interest.

In order to compare the effects of enzymes and toxins Morgenroth immunised goats against rennet and was thus able to make a rennet antiserum, which had a strong inhibiting effect upon the activity of rennet. That this inhibiting effect was due to antibodies, formed in the antiserum during the immunisation, and not to some substance in the normal goat serum, is demonstrated by Morgenroth in the following manner. Fresh milk and milk mixed with normal goat serum require exactly the same amount of rennet for their coagulation, whereas milk mixed with the antiserum in the same proportions as milk with normal goat serum only coagulates in presence of a much greater quantity of enzyme. Before Morgenroth's investigations were carried out, Hammarsten and Rödén [1887] had shown that a similar inhibiting effect may be obtained when milk is mixed with normal horse serum. In addition to this, Briot [1900] investigated the blood serum of other animals and showed that the serum of all the species examined had a more or less distinct effect on the coagulating power of rennet. In addition Briot immunised rabbits with rennet and obtained an antiserum similar to that which Morgenroth had made in goats.

In later investigations on the reaction of rennet and its antibodies the preparation of an antiserum has, however, not been attempted, because normal horse serum has a strong inhibiting effect on rennet, which was supposed to be due to a true antibody, and this obviated the necessity of preparing one. Korschun adopts this view in his publication of 1902. His

researches, which are of considerable interest, bring him to the conclusion that normal horse serum contains two substances with inhibiting action on rennet, one having a large range of action, from 20° – 37° , and the other being especially active at 37° . Korschun calls this second substance "pseudo-antilab" (pseudo-antirennet).

A more detailed account of a few of Korschun's experiments will show what led him to accept the above-mentioned theory. In order to determine the coagulating power of milk, Korschun uses the quantitative method, introduced by Morgenroth, which is undoubtedly the most reliable we have. In contradistinction to the others, this method does not indicate the coagulating power of the milk as a function of the time needed for the coagulation of a certain quantity of milk by a constant quantity of rennet, but by the minimal amount of the enzyme, which is sufficient to coagulate this quantity of milk. This method, however, cannot give reliable results, when the coagulation of the milk, that is, the transformation of the colloid caseinogen into precipitated casein is brought about in the usual way, i.e. by heating the milk-rennet complex to about 35° until the transformation has taken place. For by this heating a rather considerable amount of rennet may be destroyed without it having any effect on the caseinogen. Morgenroth's method therefore makes the transformation of the caseinogen take place at lower temperatures (0° – 8°), which do not injure the rennet, so that even very small quantities may remain active for a long time. At the low temperature, however, the casein formed is not precipitated and it is therefore necessary to raise the temperature of the milk-rennet complex to about 35° , when all the caseinogen has been transformed. At 35° the precipitation takes place rapidly, often instantaneously. The transformation of caseinogen into casein proceeds only slowly at 0° – 8° , and to ensure that the reaction should come to an end, the milk-rennet complex has to be kept at low temperature for 12–16 hours.

Morgenroth's quantitative method is very exact, and the same milk tested at various times always indicates the same coagulating power, which is by no means the case, when this power is determined by the time which is taken for the coagulation of a certain quantity of milk with a constant quantity of rennet.

Korschun's method of testing the power of his serum is also much more reliable than the methods previously used. In these earlier methods various quantities of serum and milk were mixed and the time noted which a certain quantity of rennet required for coagulating the mixture of milk and serum at the optimal temperature of the reaction (i.e. 35°). The inhibiting power of

the serum was then expressed by the difference between the time taken for the above-mentioned process and the time required for the coagulation of the same quantity of milk without serum by the same amount of rennet. Such a method, however, only gives quite a rough idea of what really happens, because by mixing serum and milk, both these substances act on the rennet, when this is being added to the mixture, and a part of the enzyme will therefore be adsorbed by the milk, and thus rendered incapable of acting with the serum. Hedin proved, in fact, some years later, that serum has the greatest inhibiting effect on rennet, when the serum and rennet are previously mixed, and milk added to them only when the action of the serum has come to an end. The same way of testing the action of serum on rennet is used in Korschun's experiments. Increasing amounts of rennet solution are added to a constant quantity of serum, and after this mixture has been kept for 15 minutes at 20° , 10 cc. of milk are added. The samples are kept all night (12-16 hours) in the ice room, and then moved to 35° in order to determine in which samples the caseinogen of the milk has been transformed into casein. The tube, which contains the greatest quantity of rennet, and is still liquid, indicates the limit of the inhibiting power of the serum. The degree of this inhibiting power is given by the ratio of rennet used to the smallest amount of enzyme, just sufficient to coagulate 10 cc. of the same milk without serum. For example, if 10 cc. of normal horse serum are able to neutralise the effect of 0.03 cc. of a rennet solution on 10 cc. of milk, whilst 0.0006 cc. rennet is sufficient to coagulate 10 cc. of the same milk without serum, then the inhibiting power of this serum is equal to 50.

Using the above-mentioned method Korschun examines the inhibiting power of fresh normal horse serum, of dialysed and of heated horse serum, and finds that dialysis does not decrease the inhibiting power. By heating the serum, however, its effect is lost. He shows, moreover, that a horse antiserum, made by immunising a goat against horse serum, neutralises the inhibiting effect of the normal horse serum. From these facts he concludes that the inhibiting effect of the normal horse serum is due to a true antibody.

Experimenting with horse serum Korschun observed that this serum neutralises more rennet than might be expected, when the rennet-serum complex is kept at 35° or 40° instead of at 20° , and that the increase of the inhibiting power at 37° - 40° is greater, up to a certain point, the longer the rennet-serum mixture is kept at the higher temperature. At 20° on the contrary no such increase in the inhibiting faculty seems to take place. He assumes therefore that in addition to the true antibody, the normal horse

serum contains a second inhibitory substance, the pseudo-antirennet, which is only active at higher temperatures.

As the dialysed serum does not show the greater effect on rennet at higher temperatures, which is shown by normal horse serum, the pseudo-antirennet must according to Korschun be dialysable. On the other hand, a heated horse serum has a strong inhibiting effect at 37° and no effect at 20°; the pseudo-antirennet appears therefore to be highly thermostable.

Hedin's researches point in quite another direction than Korschun's. In a number of publications he discusses the inhibiting influence of different substances on the activity of trypsin and rennet. In regard to rennet he points out that the intensity of the inhibiting effect depends on the order in which rennet, serum and milk are mixed. As already mentioned, he finds that the greatest effect is obtained when rennet and serum are first mixed, and the milk added to this complex, when the action of the serum has been brought to an end. Furthermore, Hedin shows that the longer the complex of rennet and serum is kept, before the milk is added, the greater up to a certain point is the amount of rennet neutralised, and that the maximum amount of the enzyme, which the serum is capable of destroying under the conditions observed, is different for different temperatures, the serum being more active at a higher than at a lower temperature. The concentration of the serum, however, does not seem to have any influence at all on the amount of rennet destroyed, and the inhibiting effect therefore is equally intense, whether the serum used is diluted or not.

Particular interest attaches to Hedin's observation that charcoal affects the rennet in the same way as normal horse serum, thus tending to indicate that the inhibiting effect of this serum is of a less complicated nature than was supposed by Korschun, and that it may possibly be due to a single character of the serum—its adsorbing quality. The further question then arises as to whether the inhibiting effect of the artificially produced rennet antiserum is of a nature similar to that of the normal horse serum, or due to the presence of a true antibody, formed in the serum during the immunisation.

In the experiments discussed below I have examined the inhibiting effect of normal horse serum, in order if possible to obtain agreement between Korschun's and Hedin's theories, which, as already shown, differ in various respects. At the same time the effect of the antirennet of normal horse serum has been compared with the inhibiting power of the antirennet formed in a rabbit serum after an immunisation, with the view of establishing their identity or otherwise.

I. RESEARCHES ON THE INHIBITING SUBSTANCES OF NORMAL HORSE SERUM.

(a) *The true antibody of horse serum and its mode of action.*

The capacity of neutralising a maximum amount of rennet in a very short time (a few minutes) is, according to Korschun, one of the most distinct qualities of the true antirennet, found in normal horse serum. Korschun therefore determines the inhibiting effect of this antibody by mixing horse serum and rennet solution, keeping the complex at 20° for 15 minutes, and measuring the remaining amount of active rennet.

In contradistinction to the action of the true antibody, the inhibiting effect of the so-called pseudo-antirennet only takes place after some time. For the determination of the effect of this substance therefore it is necessary to keep serum and rennet in contact with each other for at least three hours.

From Korschun's work it is evident that the true antibody is fully active at 20°, whereas the pseudo-antirennet does not show any distinct inhibiting effect at this temperature. Furthermore, as the effect of the true antibody does not increase proportionally to the time during which the serum acts on the rennet, it might be expected that the normal horse serum would behave similarly at 20°.

Experiment 1. (Table I.)

In this, as in all other experiments mentioned in this paper, Morgenroth's quantitative method has been used to determine the amount of rennet not neutralised by the serum in question. 10 cc. of milk were added to the rennet-serum complex, after this had been kept for the time required at 20°. The samples were then placed at a low temperature (0°) over night, and the following morning heated in a water-bath to 35°. The caseinogen of the milk was regarded as unaltered in those samples which remained liquid after half an hour at 35°, i.e. in such samples there had not been rennet enough left to transform the caseinogen into casein.

The minimal quantity of rennet solution required for coagulating 10 cc. of the milk used was 0.0015 cc.

The rennet solution in question was a standard solution, made at The Swiss Institute for Dairy Bacteriology, Liebefeld near Berne, according to Morgenroth's instructions. In cases where the amount of rennet used was less than 0.015 cc. the standard solution was diluted (1-10) with saline solution.

The milk used for the experiments was a mixed milk saturated with chloroform, and curdling as mentioned above by the addition of 0.0015 cc. of rennet.

The horse serum in question was a normal horse serum, the durability of which was increased by the addition of some thymol crystals.

All experiments were made in test-tubes, the inner wall of which was covered with a thin film of paraffin in order to prevent alkali from the glass affecting the rennet-serum complex. All liquids used for the experiments were also kept in paraffined tubes, or tubes of Jena-glass.

Table I shows the inhibiting power of normal horse serum at 20°.

TABLE I.

The maximum amount of rennet neutralised by 0.1 cc. of normal horse serum	Time during which the rennet-serum complex is kept at 20° before milk is added to it								
	0 min.	15 min.	1 hour	2 hours	3 hours	4 hours	5 hours	6 hours	7 hours
	0.04 cc.	0.04 cc.	0.06 cc.	0.07 cc.	0.08 cc.	0.09 cc.	0.09 cc.	0.09 cc.	0.09 cc.

The comparatively large amount of rennet neutralised at the moment of admixture, and which is not increased within the following 15 minutes, represents an actual change within this short period, and is not due to a continuation of the process when the samples were stored at 0° over night. The quantity of rennet neutralised at 0° is actually very small. An experiment, in which liquids, glasses, pipettes, etc., were kept at 0°, showed that the greatest quantity of rennet, neutralised by 0.1 cc. of normal horse serum under these circumstances, amounted to 0.005 cc.

Table I shows that the time during which the serum acts on rennet is not without importance for the inhibiting power of normal horse serum at 20°. This agrees fully with Hedin's observations. According to Korschun's views, however, the objection may be raised, that the effect of the pseudo-anti-rennet sets in at 20°. To examine the inhibiting power of the true antibody in the horse serum, it is therefore necessary to use a method other than that employed in Experiment 1.

Korschun has shown that the two inhibiting substances in the normal horse serum can be separated by dialysing the serum. As distinct from the true antibody, the pseudo-antirennet is dialysable. A dialysed horse serum should therefore give a measure only of the genuine antibody's mode of action.

As seen in Table I normal horse serum instantaneously neutralises a considerable quantity of rennet—0.04 cc. This quantity undoubtedly is

neutralised by the true antibody. A dialysed serum should therefore be capable of destroying a corresponding quantity of rennet in an equally short time, and thereby have reached the maximum of its inhibiting power. As can be seen from Tables II and III, this is by no means the case.

In these tables the results are given of two experiments, made with two samples of horse serum, the one dialysed longer than the other. For the experiment described in Table II, 10 cc. of the horse serum were dialysed for 48 hours in an animal membrane ("Fischblase") against running tap-water. To avoid bacterial action some thymol crystals were added to the serum. During the dialysis the volume of the serum increased to 14 cc. The precipitated englobulin was separated from the dialysed serum by centrifuging. This englobulin was also tested for inhibiting power, but proved to be without effect. Korschun has made the same observation. Half of the dialysed serum was used for the experiment, mentioned in Table II, while the remaining part—7 cc.—was brought back into the membrane and dialysed for further 48 hours. The volume of this second part thereby increased to 10.5 cc. This serum was used for the experiment in Table III.

In Fig. 1 the inhibiting powers of the dialysed sera are graphically shown, and compared with the inhibiting power of normal horse serum at 37° (Table IV). Like all the curves in this paper, those in Fig. 1 indicate the effect of the inhibiting substance in question, expressed by the ratio between the maximum amount of rennet, neutralised by the inhibiting substance, and the minimal quantity of rennet (i.e. 0.0015 cc.), required for the coagulation of 10 cc. of the milk used, without any addition of serum.

TABLE II.

The maximum amount of rennet neutralised by 0.14 cc. of a horse serum, dialysed for 48 hours	Time during which the rennet-serum complex is kept at 37° before milk is added to it									
	0 min.	15 min.	1 hour	2 hours	3 hours	4 hours	5 hours	6 hours	7 hours	
	0.02 cc.	0.08 cc.	0.12 cc.	0.14 cc.	0.16 cc.	0.16 cc.	0.16 cc.	0.16 cc.	0.16 cc.	0.16 cc.

As seen above, 0.14 cc. horse serum dialysed for 48 hours is equal to 0.1 cc. of normal horse serum.

TABLE III.

The maximum amount of rennet neutralised by 0.21 cc. horse serum, dialysed for 96 hours ..	Time during which the rennet-serum complex is kept at 37° before milk is added to it									
	0 min.	15 min.	1 hour	2 hours	3 hours	4 hours	5 hours	6 hours	7 hours	
	0.015 cc.	0.06 cc.	0.11 cc.	0.13 cc.	0.15 cc.	0.15 cc.	0.15 cc.	0.15 cc.	0.15 cc.	0.15 cc.

0.21 cc. of the horse serum dialysed for 96 hours corresponds to 0.1 cc. normal horse serum.

The curves in Fig. 1 show perhaps more clearly than the corresponding tables that the period for which rennet and dialysed serum are kept before the milk is added to the complex is of decisive importance for the inhibiting power of these sera. The dialysis therefore does not differentiate between the true antibody and the pseudo-antirennet with the loss of the latter, but decreases

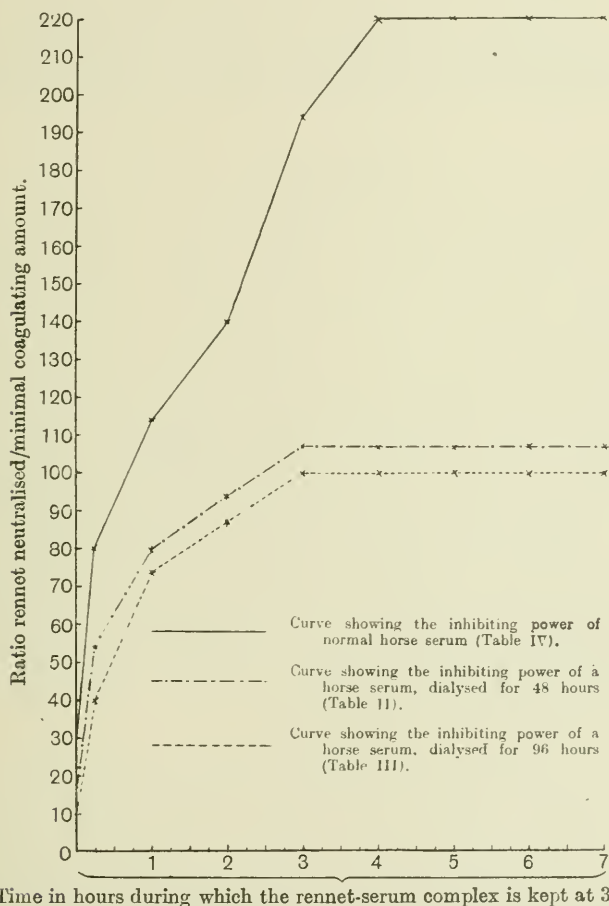


Fig. 1. Showing the inhibiting power at 37° of normal and dialysed horse serum.

the total inhibiting power of the serum, a decrease which is evidently greater the longer the dialysis lasts.

Even if the above data do not give any direct information as to the nature of the true antibody of horse serum, two conclusions may be drawn from them. Firstly, that this body cannot be a true antibody in the strict sense of the term, since the power of a true antibody is not decreased by dialysis

of the serum in which it is contained¹. And secondly, they show that there is no such contrast between "true antibody" and "pseudo-antirennet" in their relations to dialysis, as Korschun supposed, which to him was one of the essential arguments for regarding them as two different substances.

(b) *Pseudo-antirennet; its mode of action and its nature.*

When a normal horse serum acts on rennet at 37° its inhibiting effect is much greater than at 20°, as shown in the following table (see Fig. 1).

TABLE IV.

Time during which the rennet-serum complex is kept at 37° before milk is added to it										
	0	15	1	2	3	4	5	6	7	
	min.	min.	hour	hours	hours	hours	hours	hours	hours	
the maximum amount of rennet neutralised by 0.1 cc. of normal horse serum	0.04 cc.	0.12 cc.	0.17 cc.	0.21 cc.	0.29 cc.	0.33 cc.	0.33 cc.	0.33 cc.	0.33 cc.	0.33 cc.

This observation also agrees with Hedin's statement that the inhibiting power of the serum increases up to a certain equilibrium proportionally, not only to the time, but also to the temperature at which the serum acts on the rennet. Korschun regards this increase in the inhibiting power as being due to the so-called pseudo-antirennet. As this body is supposed to be extremely thermostable, it ought to be separated from the thermolabile "true antibody" by heating the horse serum. An experiment made with a heated serum therefore should show the effect of the pseudo-antirennet better than the normal horse serum was able to do.

Following Korschun's instructions, an experiment like this is best made with a horse serum diluted in the proportions (1 + 5) with distilled water or tap-water, and then boiled for some time. In making the experiment, 10 cc. of the above-mentioned horse serum were therefore mixed with 50 cc. of distilled water, and the mixture kept in a boiling water-bath for one hour. The inhibiting effect of the serum dilution thus treated was determined in the usual way and found to have the following values.

¹ The only apparent cause for a decrease in the power of a true antibody would be that the dialysis had deprived the serum, in which the antibody is contained, of its content of NaCl. But even if NaCl be added to the dialysed horse serum the inhibiting power of the latter is not altered.

TABLE V.

The maximum amount of rennet neutralised by 0.6 cc. of a boiled horse serum dilution (1+5)	Time during which the rennet-serum complex is kept at 37° before milk is added to it									
	0 min.	15 min.	1 hour	2 hours	3 hours	4 hours	5 hours	6 hours	7 hours	
0.004 cc.	0.08 cc.	0.20 cc.	0.29 cc.	0.31 cc.	0.31 cc.	0.31 cc.	0.31 cc.	0.31 cc.	0.31 cc.	0.31 cc.

0.6 cc. of the boiled serum dilution contains 0.1 cc. horse serum.

The use of so large a quantity of serum dilution made the precipitation of the casein, formed in the samples by the action of the rennet, extremely difficult, as the dilution had lowered the concentration of the calcium salts in the milk too much. Normal conditions were, however easily brought about by adding some drops of a calcium chloride solution (4 %).

This experiment plainly shows that by heating the horse serum a considerable decrease is effected in the amount of rennet instantaneously neutralised by the true antibody. On the other hand, the heating does not appear to have changed the inhibiting power of the pseudo-antirennet, as the quantity of rennet neutralised at 37° by the heated serum practically agrees with what is destroyed at the same temperature by normal horse serum (Table IV). Notwithstanding this, it would be erroneous to conclude, as Korschun does, that the heating has destroyed the true antibody without hurting the pseudo-antirennet. A closer examination of the conditions, obtaining in the experiment to which Table V refers, shows that a horse serum, diluted (1 + 5) and heated to 70° for a quarter of an hour, has a higher inhibiting power at 37° than the boiled dilution and that an unheated dilution (1 + 5) has a still greater effect at the same temperature. By heating a serum dilution therefore we weaken its total inhibiting power, also that part of it which is due to the pseudo-antirennet. Tables VI and VII and Fig. 2 illustrate this.

TABLE VI.

The maximum amount of rennet neutralised by 0.6 cc. of a horse serum dilution (1+5), heated to 70° for a quarter of an hour ..	Time during which the rennet-serum complex is kept at 37° before milk is added to it									
	0 min.	15 min.	1 hour	2 hours	3 hours	4 hours	5 hours	6 hours	7 hours	
0.006 cc.	0.09 cc.	0.21 cc.	0.29 cc.	0.32 cc.	0.35 cc.	0.38 cc.	0.38 cc.	0.38 cc.	0.38 cc.	0.38 cc.

0.6 cc. of the dilution used contained 0.1 cc. horse serum.

TABLE VII.

The maximum amount of rennet neutralised by 0.6 cc. of a horse serum dilution (1+5) ..	Time during which the rennet-serum complex is kept at 37° before milk is added to it									
	0 min.	15 min.	1 hour	2 hours	3 hours	4 hours	5 hours	6 hours	7 hours	
0.04 cc.	0.12 cc.	0.27 cc.	0.33 cc.	0.37 cc.	0.40 cc.	0.42 cc.	0.42 cc.	0.42 cc.	0.42 cc.	0.42 cc.

0.6 cc. of the dilution used contained 0.1 cc. horse serum.

The heating has thus considerably damaged both the true antibody and the pseudo-antirennet, with the difference that the destruction of the first

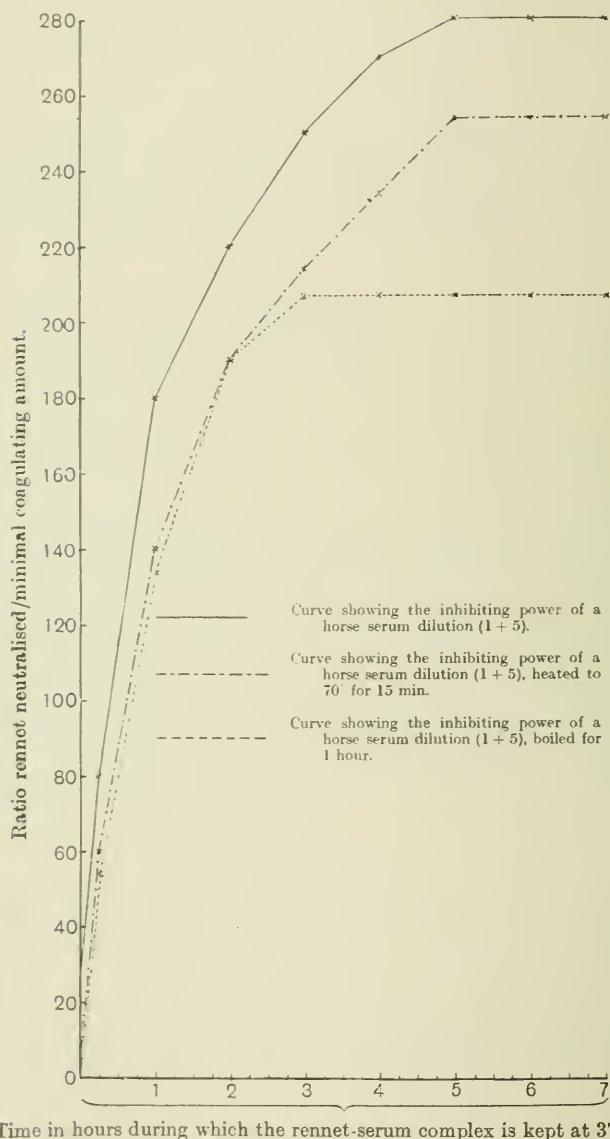


Fig. 2. Showing the inhibiting power at 37° of heated and unheated horse serum dilutions (1 + 5).

is, so to speak, complete, while the pseudo-antirennet is still active to a high degree and cannot be entirely destroyed. Then it must be assumed that the destruction of the pseudo-antirennet has practically come to an end, since a

serum dilution, boiled for one hour, gives values for its inhibiting power which are but little less than those shown by a dilution heated for a quarter of an hour to 70°. This partial destruction of the pseudo-antirennet and the increase in the inhibiting power by diluting it would hardly be explainable if the pseudo-antirennet, as supposed by Korschun, were a substance present in normal horse serum as an independent body. On consideration the above-mentioned facts rather indicate that the term pseudo-antirennet, as applied to the inhibiting power of heated and unheated serum dilutions, covers at least three properties of normal horse serum, i.e. its faculty of regulating the [H] in its dilutions, its alkalinity and its thermolability.

The importance of the first of these properties is apparent on an examination of the effect which dilution of normal horse serum has on its inhibiting power. Allusion has already been made to Hedin's statement that dilution of the normal serum does not change its inhibiting power; this is not in conformity with my own experiments. In view of the method employed by Hedin in his experiments, it is, however, impossible to attach importance to his deductions. By using Korschun's exact methods it is obvious that the inhibiting power is increased considerably by dilution of the serum. This may be seen from Tables VIII and IX, which concern two experiments made with diluted horse sera.

TABLE VIII.

The maximum amount of rennet neutralised by 0.3 cc. of a horse serum diluted (1+2)...	Time during which the rennet-serum complex is kept at 37° before milk is added to it								
	0 min.	15 min.	1 hour	2 hours	3 hours	4 hours	5 hours	6 hours	7 hours
	0.04 cc.	0.12 cc.	0.25 cc.	0.28 cc.	0.31 cc.	0.36 cc.	0.36 cc.	0.36 cc.	0.36 cc.
	0.3 cc. of the serum dilution used contained 0.1 cc. horse serum.								

TABLE IX.

The maximum amount of rennet neutralised by 0.6 cc. of a horse serum diluted (1+5)...	Time during which the rennet-serum complex is kept at 37° before milk is added to it								
	0 min.	15 min.	1 hour	2 hours	3 hours	4 hours	5 hours	6 hours	7 hours
	0.04 cc.	0.12 cc.	0.27 cc.	0.33 cc.	0.37 cc.	0.40 cc.	0.42 cc.	0.42 cc.	0.42 cc.
	0.6 cc. of the serum dilution used contained 0.1 cc. horse serum.								

This increase in the inhibiting power becomes more apparent on examination of the [H] of the normal horse serum and its dilutions. Tested electrically the [H] of the normal horse serum in question gives the value

$p_{\text{H}} = 7.99$. Diluting the serum (1 + 5) its alkalinity does not decrease. On the contrary, measured at 37° its $[\text{H}']$ gives values which vary from $p_{\text{H}} = 8.20$ to $p_{\text{H}} = 7.90$, depending on the time at which they are measured, either directly the dilution is made or later. The $[\text{H}']$ of the dilution (1 + 2) agrees, as does that of the dilution (1 + 5), with the $[\text{H}']$ of the normal horse serum.

Considering the extreme sensitiveness of rennet towards even very small quantities of alkali, it seems natural to connect the increase of the inhibiting power of the dilutions with the tendency of the serum to maintain normal $[\text{H}']$ even in its higher dilutions. Indeed we know that the amount of rennet inhibited by an alkali solution is dependent on the $[\text{H}']$ of this solution only, and not on the exact number of H- or OH-ions present. The same fact must therefore determine the action of the serum dilutions if their $[\text{H}']$ has any influence on their increased inhibiting power. From this point of view it is, however, also justifiable to suppose that a greater quantity, either of the alkaline solution or of the serum dilutions, will destroy more rennet than a smaller amount is able to do. To explain this we may suppose that 0.1 cc. of alkaline solution of a certain $[\text{H}']$ neutralises 0.02 cc. rennet solution; 0.6 cc. of the same alkaline solution should then neutralise 6×0.02 cc. = 0.12 cc. rennet. That this occurs when the alkali is kept in six tubes each containing 0.1 cc. is obvious. If 0.6 cc. of the alkali is brought into one single tube, the same amount of rennet should be inhibited, as in this case also we have 0.02 cc. rennet corresponding to each of the alkali's six units of 0.1 cc. I have tried experimentally to ascertain how far this agrees with the actual, and found that 0.6 cc. of a Na_2HPO_4 solution of a certain $[\text{H}']$ inhibits seven times more rennet than 0.1 cc. of the same phosphate solution. This result is very satisfactory, as experimental errors must always be considerable in work of this kind.

If a greater quantity of alkali is thus able to destroy more rennet than a smaller, the serum and its dilutions must do the same, as their $[\text{H}']$ is also constant; and 0.6 cc. of a serum dilution (1 + 5) must therefore inhibit more rennet than 0.3 cc. serum dilution (1 + 2), and this 0.3 cc. again more than 0.1 cc. of normal horse serum. The question whether the increase in the inhibiting power of the serum can be due to its property of maintaining normal $[\text{H}']$ in its dilutions therefore resolves itself into one, as to whether an increase in the inhibiting power, equal to that shown by the serum dilutions, can be caused by an alkaline solution of the same $[\text{H}']$ as the serum— $p_{\text{H}} = 7.99$ (at 37°)—and used in the same quantities as this.

The increase in the inhibiting power of the serum dilution (1 + 2) is equal

to 0.03, this value being the difference between the maximum amount of rennet (0.33 cc.) inhibited by 0.1 cc. of normal horse serum at 37° and the greatest quantity (0.36 cc.) destroyed at the same temperature by 0.3 cc. of the serum dilution (1 + 2).

The increase in the inhibiting power of the solution (1 + 5) is 0.06, as 0.3 cc. of the dilution (1 + 2) inhibits 0.36 cc. rennet, and 0.6 cc. of the dilution (1 + 5) inhibits 0.42 cc.

As regards the alkaline solution, it has already been pointed out that the increase in its inhibiting power is proportional to the amount of solution used. 0.3 cc. alkaline solution of a certain $[H]$ therefore inhibits three times as much rennet as 0.1 cc. of the same solution. 0.1 cc. alkali of the concentration required¹ inhibits 0.04 cc. rennet, and 0.3 cc. of this alkali therefore ought to inhibit $3 \times 0.04 = 0.12$ cc. rennet, giving an increase in the inhibiting power equal to $0.12 - 0.04 = 0.08$. On the other hand, 0.6 cc. of the alkali in question² will inhibit $6 \times 0.04 = 0.24$ cc. rennet, which represents an increase in the inhibiting power equal to $0.24 - 0.12 = 0.12$. The experiments upon which these calculations are based are somewhat crude and the values cannot be regarded as absolutely exact. Nevertheless they suffice to show that an increase in the inhibiting power such as is shown by the serum dilutions is to be expected when they are used in increasing quantities.

With respect to the other properties which are necessary for the action of the pseudo-antirennet, the alkalinity and the thermolability of the serum, their importance is best shown by a closer examination of the inhibiting power of a boiled horse serum.

In view of the results of the above-mentioned experiments one might expect the reduction of the inhibiting power of the heated serum dilutions to be due to a decrease of their $[H]$ caused by the treatment. This, however, was not the case, as the $[H]$ of the boiled serum dilution gave roughly the same value as that shown by the unheated dilutions. Nevertheless there is an important relation between the inhibiting power of the boiled serum and its $[H]$. This became evident by a determination of the inhibiting power of 0.6 cc. of the phosphate solution at 37° . The experimental data are given in Table X.

¹ A solution of Na_2HPO_4 and KH_2PO_4 was used, made according to Sørensen's [1909] instructions by mixing 9.75 cc. *n*/15 Na_2HPO_4 and 0.25 cc. *n*/15 KH_2PO_4 ; tested at 18° this mixture has the $[H']p_{H'} = 8.038$.

² In an experiment already mentioned 0.6 cc. phosphate solution neutralised 0.28 cc. rennet, and not 0.24 cc.

TABLE X.

	Time during which the rennet-phosphate complex is kept at 37° before milk is added to it								
	0	15	1	2	3	4	5	6	7
maximum amount rennet neutralised 0.6 cc. of a phos- phate solution with the	min.	min.	hour	hours	hours	hours	hours	hours	hours
$[P_H] = 8.038 (18^\circ) \dots$	0.003 cc.	0.04 cc.	0.19 cc.	0.23 cc.	0.28 cc.	0.28 cc.	0.28 cc.	0.28 cc.	0.28 cc.

As can be seen from the curves in Fig. 3 there is a striking agreement between the inhibiting effects of the phosphate solution and the boiled serum

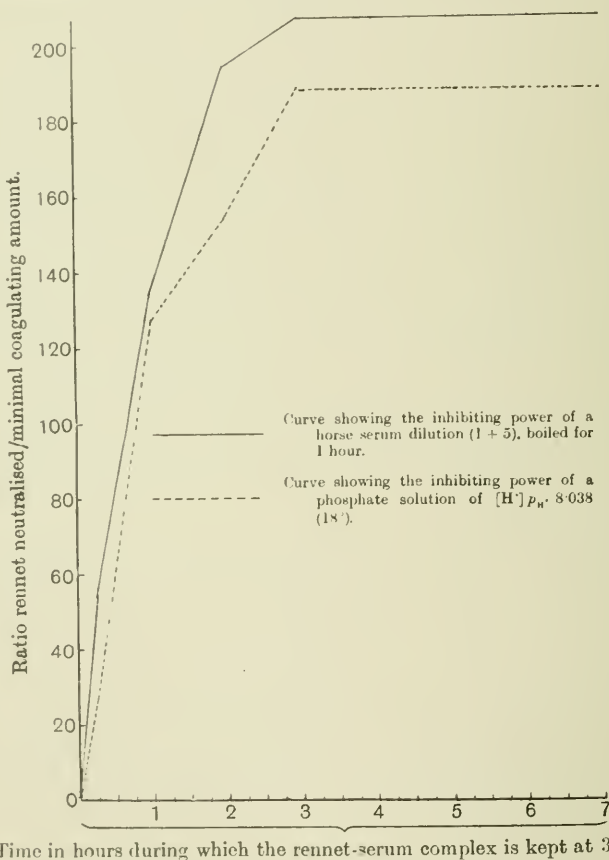


Fig. 3. Showing the inhibiting power at 37° of a boiled horse serum dilution (1+5) and of a phosphate solution of nearly the same $[H^+]$.

dilution. There appears no reason to doubt that this agreement is due to the fact that the inhibiting power of the boiled horse serum is an effect of its $[H^+]$ only. This will be sufficient to indicate the importance of the alkalinity

of the serum with respect to its inhibiting power. It therefore only remains to be shown that the inhibiting power is dependent also on the thermolability of the serum.

The value of the $[H]$ is clearly as important for the inhibiting power of the serum dilutions as it was found to be for the action of the boiled serum. The inhibiting power of the dilution (1 + 5), for instance, must therefore be regarded as a mixed effect of its $[H]$ and of various other supplementary properties (substances?). In addition to the effect of the $[H]$ the inhibiting power of this dilution therefore also shows the effect of these properties. As can be seen from Table XI they also relate to the pseudo-antirennet, as their inhibiting power increases proportionally to the time the serum acts on the rennet.

TABLE XI.

	Time during which the serum or the phosphate solution acts on the rennet at	0	15	1	2	3	4	5	6	7
	min.	min.	hour	hours	hours	hours	hours	hours	hours	hours
The maximum amount of rennet neutralised by 0.6 cc. of a serum dilution (1 + 5)	0.04 cc.	0.12 cc.	0.27 cc.	0.33 cc.	0.40 cc.	0.42 cc.	0.42 cc.	0.42 cc.	0.42 cc.	0.42 cc.
The maximum amount of rennet neutralised by 0.6 cc. of a phosphate solution of the same $[H]$ as the horse serum	0.003 cc.	0.04 cc.	0.19 cc.	0.23 cc.	0.28 cc.	0.28 cc.	0.28 cc.	0.28 cc.	0.28 cc.	0.28 cc.
The inhibiting effect of the supplementary properties of the horse serum	0.037 cc.	0.08 cc.	0.08 cc.	0.10 cc.	0.12 cc.	0.14 cc.	0.14 cc.	0.14 cc.	0.14 cc.	0.14 cc.

Calculations similar to that made in Table XI show that the effect of these supplementary properties of the serum dilution (1 + 5) is greatly reduced by heating it to 70°. In a boiled dilution their effect has completely disappeared. As the thermolability of the serum is thus decisive for the effect of these properties, it must also be of importance for the action of the pseudo-antirennet, since the properties in question, as shown in Table XI, are part of the pseudo-antirennet.

Some interesting light as to the nature of these supplementary properties, or substances, of normal horse serum may be obtained from the experiments already discussed. As they cannot be demonstrated in a boiled horse serum dilution, their effect is obviously due to the proteins of the serum, or perhaps to their colloid qualities. Moreover, the effect of the properties in question increases proportionally to time, and to the temperature at which the serum acts on rennet. This can be seen from Tables I and XII. Table I showed the inhibiting power of 0.1 cc. of normal horse serum at 20°. But the figures

at the same time also practically show the inhibiting effect of the supplementary properties at 20° , as the $[H]$ of 0.1 cc. of normal horse serum is hardly of any importance for the amount of rennet destroyed at this temperature. Experiments showed that 0.1 cc. of the phosphate solution neutralised 0.003 cc. rennet at 20° , an amount which does not increase even if the solution acts on rennet for several hours.

The inhibiting power of the supplementary properties at 37° is shown in Table XII.

TABLE XII.

	Time during which the serum or the phosphate solution acts on the rennet at 37°								
	0 min.	15 min.	1 hour	2 hours	3 hours	4 hours	5 hours	6 hours	7 hours
The maximum amount of rennet neutralised by 0.1 cc. of normal horse serum	0.04 cc.	0.12 cc.	0.17 cc.	0.21 cc.	0.29 cc.	0.33 cc.	0.33 cc.	0.33 cc.	0.33 cc.
The maximum amount of rennet neutralised by 0.1 cc. phosphate solution of the same $[H]$ as the horse serum	0.003 cc.	0.02 cc.	0.04 cc.	0.04 cc.	0.04 cc.	0.04 cc.	0.04 cc.	0.04 cc.	0.04 cc.
The effect of the supplementary properties of the horse serum is therefore equal to	0.037 cc.	0.10 cc.	0.13 cc.	0.17 cc.	0.25 cc.	0.29 cc.	0.29 cc.	0.29 cc.	0.29 cc.

The effect of these properties is thus considerably increased by raising the temperature at which the experiment is made.

Furthermore, some light is thrown on them from Table XII. Compared with the figures in Table XI, which showed their inhibiting power at high dilution, the figures in Table XII indicate that their effect is greater when they act on rennet in concentrated than in diluted form.

The real cause of the appearance of these properties, or substances, cannot be determined from the experiments discussed above. Korschun's "true antibody" is certainly either identical with them or part of them. Nevertheless the use of this expression for the explanation of the action of the normal serum on rennet is not to be recommended. This action cannot be due to a true antibody, but is more probably attributable to physico-chemical effects. On the other hand, Hedin's conclusion that this action is a mere adsorption must undoubtedly only be a partial explanation.

II. THE INHIBITING EFFECT OF NORMAL RABBIT SERUM ON RENNET.

As can be seen from the following table the action of normal rabbit serum on rennet has the same characteristics as that of normal horse serum, with the difference merely that its effect is distinctly weaker.

TABLE XIII.

No.	Material	Temp.	Vol. of serum	Maximum amount (in cc.) of rennet neutralised when the rennet-serum complex is kept for the following times before milk is added to it								
				0	0.25	1	2	3	4	5	6	7 hours
1	Normal rabbit serum	20°	0.1 cc.	0.004	0.008	0.01	0.015	0.03	0.03	0.03	0.03	0.03
2	Normal rabbit serum	37°	0.1 cc.	0.004	0.025	0.085	0.11	0.14	0.17	0.17	0.17	0.17
3	Normal rabbit serum dialysed for 48 hours through an animal membrane	37°	0.35 cc. of the dialysed serum (=0.1 cc. undilysed serum)	0.0015	0.02	0.07	0.09	0.15	0.17	0.17	0.17	0.17
4	Rabbit serum diluted (1+5)	37°	0.6 cc. diluted serum (=0.1 cc. normal serum)	0.004	0.025	0.09	0.13	0.16	0.18	0.21	0.21	0.21
	Rabbit serum diluted (1+5) and heated to 70° for 0.25 hour	37°	0.6 cc. diluted serum (=0.1 cc. undiluted serum)	0.0015	0.006	0.12	0.15	0.15	0.15	0.15	0.15	0.15

The experiments from which these numbers are derived were arrived at in the same way as those dealing with the inhibiting power of normal horse serum. The same rabbit serum was used for all experiments. In addition two other rabbit sera were tested, one of which showed a lower inhibiting power than that used for the experiments quoted. The intensity of the inhibiting power thus varies not only with different species of animals, but also with individuals of the same species, a fact which has already been recognised.

III. THE INHIBITING POWER OF A RENNET ANTISERUM.

The question of the possible identity of artificial antirennet and that found in normal horse serum has already been referred to in the introduction. Previous researches, Korschun's and Morgenroth's [1900] especially, appeared to point in this direction, at any rate as far as those rennet antisera were concerned, which were made by immunising animals, e.g. goats or rabbits, with a true rennet enzyme. The antirennet of the normal serum, or at least a part of it, was regarded as a true antibody, formed in the serum of the animal in question by autoimmunisation with rennet, originating from the mucous membranes of its stomach. As the results of the experiments mentioned in

this paper affect this conception of the inhibiting effect of normal horse serum in various respects, it appeared of interest to ascertain whether the action of the rennet antiserum could be explained in the same manner as that of the normal serum, or whether in this antiserum there occurred a typical antibody with properties similar to those claimed by Korschun for the antibody of normal horse serum.

The rennet antiserum, employed in the experiments discussed below, was obtained in the following manner.

The immunisation was accomplished in The Swiss Institute for Dairy Bacteriology, Liebefeld near Berne. As antigen a pure rennet powder (Merck) with the coagulating power $1-1.5 \times 10^6$ was used. The animals were injected with an extract of this rennet powder in the strength of 1.0 g. of rennet powder to 10 cc. of sterile distilled water. For each injection, given every other day, a fresh quantity of extract was made. At the beginning of the treatment the rabbits used for the immunisation did not show any loss of weight, but later on, after the seventh injection, a rather heavy fall could be noticed. Two of the rabbits died at the end of the treatment with typical anaphylactic symptoms. The subcutaneous injection caused a somewhat considerable local infiltration. Contrary to what was expected, the rennet antiserum was found to be very durable. Even at the end of a year its inhibiting power was entirely unimpaired.

Unfortunately it has been necessary to limit the number of experiments originally planned with this antiserum, as the quantity available was altogether inadequate, especially as each experiment had to be carried out two or three times.

It is interesting to note that the inhibiting power of the rennet antiserum at 20° and 37° agrees well with the properties claimed by Korschun for a serum containing a true antirennet. See Table XIV.

TABLE XIV.

The inhibiting power of rennet antiserum at 20° and 37° .

	Time during which the rennet-serum complex is kept before milk is added to it								
1.	0	15	1	2	3	4	5	6	7
	min.	min.	hour	hours	hours	hours	hours	hours	hours
The maximum amount of rennet neutralised by 0.1 cc. of a rennet antiserum, at 20°	0.03 cc.	0.03 cc.	0.03 cc.	0.03 cc.	0.03 cc.	0.03 cc.	0.03 cc.	0.03 cc.	0.03 cc.
2.									
The maximum amount of rennet neutralised by 0.1 cc. of a rennet antiserum, at 37°	0.03 cc.	0.045 cc.	0.065 cc.	0.09 cc.	0.115 cc.	0.13 cc.	0.13 cc.	0.13 cc.	0.13 cc.

At 20° the antiserum thus neutralises a considerable quantity of rennet, which does not increase even if the rennet-antiserum complex is kept for seven hours at 20°. Furthermore, the effect of the true antirennet, i.e. the capacity of the antiserum for destroying a maximum amount of rennet in a very short time (a few seconds), has considerably increased owing to the immunisation, whereas the effect of the pseudo-antirennet at 37° is still unaltered, as the difference in the inhibiting power between the pseudo-antirennet of the normal rabbit serum and that of the antiserum (Tables XIII, No. 2, and XIV, No. 2) is no doubt due to individual differences in the inhibiting power of sera of the same species, and not to a decrease in the effect of the pseudo-antirennet owing to the dialysis.

At this point, however, the agreement between the effect of the antiserum and the properties claimed by Korschun for the true antirennet ceases. Testing the effect of the antiserum under other conditions, the agreement rather seems to have been accidental. Dialysis, e.g., causes a decrease in the entire inhibiting power of the antiserum (Table XV), just as was the case with the dialysed horse serum.

TABLE XV.

The maximum amount of rennet neutralised by 0.22 cc. of a rennet antiserum dialysed for 48 hours	Time during which the rennet-serum complex is kept at 37° before milk is added to it									
	0 min.	15 min.	1 hour	2 hours	3 hours	4 hours	5 hours	6 hours	7 hours	
	0.01 cc.	0.03 cc.	0.05 cc.	0.08 cc.	0.08 cc.	0.08 cc.	0.08 cc.	0.08 cc.	0.08 cc.	0.08 cc.

3 cc. of antiserum were dialysed for 48 hours, its volume thereby increasing to 6.5 cc. 0.22 cc. of the dialysed antiserum therefore is equal to 0.1 cc. untreated antiserum.

This decrease in the inhibiting power of the antiserum is not consistent with the presence of a true antibody in this serum.

Similarly, it is noteworthy that the effect of the pseudo-antirennet is increased far more by diluting the antiserum (Table XVI) than by diluting normal rabbit serum (Table XIII, No. 4). This would hardly be the case if the pseudo-antirennet, as assumed above, had not been altered by the immunisation.

TABLE XVI.

The maximum amount of rennet neutralised by 0.6 cc. of a rennet antiserum diluted (1 + 5)	Time during which the rennet-serum complex is kept at 37° before milk is added to it									
	0 min.	15 min.	1 hour	2 hours	3 hours	4 hours	5 hours	6 hours	7 hours	
	0.01 cc.	0.06 cc.	0.20 cc.	0.22 cc.	0.23 cc.	0.24 cc.	0.24 cc.	0.24 cc.	0.24 cc.	0.24 cc.

0.6 cc. of the diluted antiserum contained 0.1 cc. antiserum.

The increase in the effect of the pseudo-antirennet is more explicable if viewed from the same standpoint as the changes in the inhibiting power of normal horse serum caused by dilution. Unfortunately, the experiments made with the antiserum had not left a sufficient quantity for a determination of its $[H']$. Using Sørensen's indicator method it was, however, found that the antiserum was more alkaline than normal rabbit serum, but less alkaline than normal horse serum¹. Dilutions of the antiserum therefore have a smaller $[H']$, i.e. a higher alkalinity, than dilutions of normal rabbit serum, as the sera are able to maintain normal $[H']$ even in high dilutions. As a matter of course the dilutions of the antiserum therefore must destroy more rennet than dilutions of normal rabbit serum.

There is another peculiarity in the inhibiting effect of the antiserum at 20°, which is not so easily explained as the changes in the action of the pseudo-antirennet. As can be seen from Tables XIII, No. 1, and XIV, No. 1, the maximum amount of rennet neutralised by normal rabbit serum at 20° is equal to that destroyed by the antiserum at the same temperature. The velocity of reaction of the destruction is, however, very different for the two sera, the antiserum neutralising the maximum amount in a very short time, normal rabbit serum, on the contrary, in four hours only. As regards the diluted antiserum, the velocity of reaction is less than that of the antiserum itself (Table XVI).

Changes in the velocity of reaction, such as are shown by the antiserum, are well known from the study of the action of adsorbing bodies [Bayliss, 1906]. As was the case for the antibody, these changes are proportional to the concentration of the adsorbing body; adsorption increasing with the concentration. The peculiarity of the inhibiting effect referred to above is therefore possibly due to an increase in the concentration of some substance in the serum during the immunisation.

The researches on the inhibiting power of the antiserum here discussed do not favour the belief that the action of the rennet antiserum is of a special nature. On the contrary, they appear to indicate that this action cannot be due to the presence of a true antibody formed in the serum during immunisation.

¹ This is remarkable, as in his researches on the $[H']$ of the normal serum Michaelis [1914] points out that changes in the normal $[H']$ of the serum are extremely infrequent, at least as far as an increase is concerned.

CONCLUSIONS.

1. The inhibiting effect of normal horse serum on the coagulating power of the rennet enzyme is not, as supposed by Korschun, due to two inhibiting substances, the so-called true antibody and the pseudo-antirennet. Neither can Hedin's explanation of this effect as a mere adsorption be regarded as fully satisfactory.

2. It can be shown furthermore that the $[H]$ of normal horse serum is highly important for its inhibiting power.

3. Normal rabbit serum shows the same characteristics in its effect on rennet as normal horse serum.

4. The inhibiting power of a rennet antiserum made by immunising rabbits with rennet enzyme can hardly be due to the presence of a true antibody, as this serum also tends to show the characteristics of normal horse serum.

The author takes the opportunity of expressing his thanks to Prof. R. Burri, head of The Swiss Institute for Dairy Bacteriology, Liebefeld near Berne, who placed the standardised rennet solution at his disposal. He is also very indebted to Dr E. Atkin, of the Lister Institute, for the determinations of $[H]$ mentioned in this paper.

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THE INFLUENCE OF TEMPERATURE, SUBMERSION AND BURIAL ON THE SURVIVAL OF EGGS AND LARVAE OF CIMEX LECTULARIUS.

By A. W. BACOT,

Entomologist to the Lister Institute.

These experiments were performed in response to a question submitted to me from the Royal Sanitary Institute. The point at issue was the possibility of eggs of the common bed-bug, *Cimex lectularius*, surviving the process of house-destruction, when the plaster from old walls, on which eggs had been laid, was broken down and remixed with fresh mortar for making the partitions of rooms in new tenements; such survival having been given as an explanation for previously unoccupied houses being infested with bugs.

Methods.

The ova used in the following tests were obtained by placing twenty to thirty adult specimens of *C. lectularius* in a 1½-inch glass-bottomed entomological box. The open top of the box was covered with fine gauze, through which the bugs were allowed to feed liberally each day. When feeding was not in progress the box containing the insects was kept in an incubator at 75°F. A slip of cloth was used as a lining to the box to afford foothold. As the great majority of the eggs were laid on the cloth this plan afforded an easy means of dividing up batches of eggs into two or more lots, so that controls could be obtained without any disturbance to the eggs. In order to get large numbers for some of the experiments the eggs were in certain instances allowed to accumulate for from three to five days before removal.

In experiments in which the eggs were submerged in water or buried in sand, those laid on cloth alone were used, but where the test was one of temperature alone, eggs laid on the sides of the boxes were occasionally utilised. In the case of the experiment with plaster the eggs were carefully detached from the sides of the box and cloth on which they had been laid. Control eggs were always taken from the same batch as those experimented with and all control eggs were kept from the time of laying in an incubator at 75°F.

The attached table shows the number of eggs in each batch, together with the date of laying. Batches are lettered from (a) to (r), and as the batch letter is quoted against each experiment, it is possible to follow the history and find the percentage hatching in different portions of the same batch which were subjected to different conditions.

*Record of Eggs Laid and Nature of Experiments for which the various
Batches were used.*

Designation of Batch.	Number of Eggs in Batch.	Dates between which the various Batches were laid.	Numbers of Control Speci- mens.	Numbers of Specimens used in Experiments.		Submergence in			
				Cold.	Heat.	Water.	Wet Sand.	Dry Sand.	Lime Water.
a ..	90	15-21 Nov. '13 ..	—	45	45	—	—	—	—
b ..	75	21-23 „ ..	—	37	38	—	—	—	—
c ..	61	23-27 „ ..	25	—	—	11	25	—	—
d ..	87	27 Nov.-1 Dec. '13	29	—	—	—	—	58	—
e ..	45	1-4 Dec. '13 ..	15	—	—	30	—	—	—
f ..	93	4-9 „ ..	15	28	50	—	—	—	—
g ..	33	9-15 „ ..	13	20	—	—	—	—	—
h ..	18	24-25 March '13 ..	4	14	—	—	—	—	—
i ..	18	25-26 „ ..	2	16	—	—	—	—	—
j ..	16	26-27 „ ..	5	—	—	11	—	—	—
k ..	75	27-30 „ ..	10	46	—	19	—	—	—
l ..	32	30-31 „ ..	12	—	—	20	—	—	—
m ..	54	31 Mar.-2 Apr. '14	20	—	—	—	34	—	—
n ..	17	2-3 April '14 ..	6	—	—	11	—	—	—
o ..	70	3-6 „ ..	11	41	—	18	—	—	—
p ..	25	6-7 „ ..	8	—	—	17	—	—	—
q ..	73	7-10 „ ..	13	60	—	—	—	—	—
r ..	50	1-6 May '14 ..	19	—	—	—	—	—	31

Temperature experiments.—Ova laid on cloth were enclosed in an entomological box (of card with a glass bottom), and this was nested in one of a larger size to avoid any possibility of escape. In cases where eggs laid on the sides of the card box were used, this box was enclosed in a larger one.

Submersion experiments.—Pieces of cloth on which the eggs were laid were fastened by one corner at the bottom of a waxed card jar and water was then poured in until the eggs were three or four inches below the surface.

Burial in sand.—A card jar was half filled with silver sand, the cloth with eggs attached was then placed on the surface and covered with sand to the depth of at least one inch. For the wet sand tests the jar was flooded with water, and the excess poured off. After the period of test the cloth was removed from the sand and placed in a box preparatory to hatching. As it was usually found that a few of the eggs became detached, the sand surrounding the cloth which contained the missing eggs, was placed in a second box. Washing off the surface on which they had been laid had no effect on the eggs, which hatched at the same time as those remaining attached to the cloth.

Plaster.—The plaster of Paris used in this experiment was mixed with lime-water. The surface was made very wet, so that the eggs might become partially embedded. They were scattered over the wet surface and became too firmly attached when the plaster set to allow of removal with a camel's hair brush.

Experiments with newly hatched bugs.—The insects used in these experiments were taken from among those hatching in the control boxes; they were not, therefore,

all from the same batch of eggs, but the eggs had all been subjected to similar conditions prior to hatching.

Ova subjected to cool conditions.

34 eggs of batch (*q*), kept between 40° and 55° F. (average temperature about 48° F.) for 12 days and then transferred to 75° F.—32 hatched=94 per cent. (Control, 92 per cent. hatched.)

26 eggs of batch (*q*), kept between 40° and 55° F. (average temperature about 48° F.) for 20 days and then transferred to 75° F.—16 hatched=61 per cent. (Control, 92 per cent. hatched.)

46 eggs of batch (*k*), kept between 40° and 55° F. (average temperature about 48° F.) for 31 days and then transferred to 75° F.—25 hatched=54 per cent. (Control, 100 per cent. hatched.)

A marked feature of the last two experiments was the number of deaths during the act of emergence from the egg; in the last experiment no less than 10 (21 per cent.) failed to get free from the egg-shell.

Ova subjected to cold.

20 eggs of batch (*f*) kept at 28° F. for 6½ hours and then transferred to 75° F.—28 hatched=100 per cent. (Control, 93 per cent. hatched.)

20 eggs of batch (*g*) kept between 28° and 32° F. for 24 hours and then transferred to 75° F.—19 hatched=95 per cent. (Control, 84 per cent. hatched.)

14 eggs of batch (*h*) kept between 28° and 32° F. for 2 days and then transferred to 75° F.—13 hatched=93 per cent. (Control, 100 per cent. hatched.)

16 eggs of batch (*i*) kept between 28° and 32° F. for 5 days and then transferred to 75° F.—4 hatched=25 per cent. (Control, 100 per cent. hatched.)

41 eggs of batch (*o*) kept between 28° and 32° F. for 8 days and then transferred to 75° F.—10 hatched=24 per cent. (Control, 100 per cent. hatched.)

45 eggs of batch (*a*) kept between 28° and 32° F. for 10 days and then transferred to 75° F.—*none hatched*. (Control, see batch (*a*) under Heat.)

8 eggs of batch (*b*) kept between 28° and 32° F. for 10 days and then transferred to 75° F.—*none hatched*. (Control, see batch (*b*) under Heat.)

29 eggs of batch (*b*) kept between 28° and 32° F. for 15 days and then transferred to 75° F.—*none hatched*.

Ova subjected to heat.

45 eggs of batch (*a*) kept at 98° F., humidity .25;—41 hatched=91 per cent.

38 eggs of batch (*b*) kept at 98° F., humidity .25;—33 hatched=100 per cent.

50 eggs of batch (*f*) kept at 113° F.;—*none hatched*. (Control, 93 per cent. hatched.)

Ova submerged in water.

Between 60° and 63° F.

11 eggs of batch (*c*) submerged for 26 hours—11 hatched=100 per cent. (Control, 96 per cent. hatched.)

30 eggs of batch (*e*) submerged for 44 hours—23 hatched=76 per cent. (Control, 93 per cent. hatched.)

18 eggs of batch (*o*) submerged for 3 days—17 hatched = 94 per cent. (Control, 100 per cent. hatched.)

19 eggs of batch (*k*) submerged for 3 days—19 hatched = 100 per cent. (Control, 100 per cent. hatched.)

11 eggs of batch (*j*) submerged for 5 days—10 hatched = 91 per cent. (Control, 100 per cent. hatched.)

Between 48° and 50° F.

20 eggs of batch (*l*) submerged for 24 hours—16 hatched = 80 per cent. (Control, 91 per cent. hatched.)

11 eggs of batch (*n*) submerged for 3 days—11 hatched = 100 per cent. (Control, 100 per cent. hatched.)

*Between 28° and 32° F.**

9 eggs of batch (*p*) submerged for 24 hours—9 hatched = 100 per cent. (Control, 100 per cent. hatched.)

8 eggs of batch (*p*) submerged for 48 hours—7 hatched = 87 per cent. (Control, 100 per cent. hatched.)

Lime-Water.

50 eggs of batch (*r*) were submerged in lime-water (saturated solution of CaH_2O_2) for 46 hours at 63° F. and were then kept at 75° F.;—*none hatched.* (Control, 100 per cent. hatched.)

Ova buried in sand.

Dry.

58 eggs of batch (*d*). The pot in which the eggs were buried was kept in a shed without doors, open towards the north from 1st to 5th December, 1913. Temperature about 43° to 47° F. The eggs were then transferred to the laboratory, at from 60° to 63° F.—58 hatched = 100 per cent. (Control, 76 per cent. hatched.)

Saturated.

25 eggs of batch (*e*). The pot in which the eggs were buried was kept in the same shed as mentioned above from 27th November to 1st December. Temperature about 43° to 50° F. The eggs were then transferred to the laboratory at 60° to 63° F.—24 hatched = 96 per cent. (Control, 96 per cent. hatched.)

20 eggs of batch (*m*). The pot in which the eggs were buried was kept in a cool room at from 47° to 53° F. for 4 days. The eggs were then transferred to an incubator at 75° F.—19 hatched = 95 per cent. (Control, 100 per cent. hatched.)

14 eggs of batch (*m*). The pot in which the eggs were buried was kept in a cool room between 47° and 55° F. for one week. The eggs were then transferred to an incubator at 75° F.—12 hatched = 86 per cent. (Control, 100 per cent. hatched.)

Ova set in plaster.

25 eggs of batch (*s*) were carefully detached from the sides and bottom of the box and cloth on which they had been laid. The eggs were then scattered on to a very

*The water was frozen round the eggs.

wet plaster surface (gypsum, mixed with a saturated solution of CaH_2O_2) so that they were partially embedded. All were found to be attached. 13 hatched = 52 per cent.

Experiments with larval bugs in 1st instar.

Cold conditions ; unfed. 7 unfed and 2 fed larvae were kept at between 28° and 32° F. for 2 days ; they had then lost power of movement. After half an hour at 63° F. some had already recovered their activity. They were again kept at between 28° and 32° F. for 5 days and on examination were, to all appearances, dead, but after 4 hours at 63° F. they had all but one recovered their activity. After a further subjection to between 28° and 32° F. for 25 days they were kept at 63° F., but no recovery took place.

14 unfed larvae were kept at between 28° and 32° F. for 16 days and then kept at 63° F. ; 3 made feeble movements after 2 hours, 6 were active after 24 hours, and 10 were active after 48 hours. The remaining 4 made no movement, but were probably paralysed rather than dead, as they showed no signs of shrinkage or decay after 96 hours, while their limbs reacted slightly to touch ; 9 of the 10 that recovered were alive 12 days later.

Cold conditions ; fed. 13 were allowed to feed on human blood and then kept in an entomological box at between 28° and 32° F. for 18 days. They were then transferred to 63° F. Only 3 showed any signs of life and one of these soon died ; the remaining 2 lived for 15 days. Of these 13, it was very noticeable that all those which fed to excess failed to recover. Of the 3 which revived none had had a full meal, and the 2 which survived longest had obtained but little food. Death was not, I think, due to cold, but to the humidity in an artificially cooled room. Confinement in test-tubes, even when crumpled paper is given for a foothold, produces a high mortality among young, well-fed bugs, under ordinary laboratory conditions of temperature ; while confinement in card boxes under similar conditions shows no death rate. Apparently it is necessary for bugs to be able to get rid of superfluous moisture rapidly after a heavy meal, and this is inhibited in a humid atmosphere.

Moderate conditions ; unfed. 38 unfed larvae hatched at 60° to 63° F. from eggs that had been buried for 4 days in dry sand at between 43° and 47° F. (see experiment page 114, batch (d)) were kept in the laboratory at between 60° and 65° F., and afford an excellent example of the ability of this pest to survive unfed under favourable conditions.

23 were active after 66 days	10 were active after 117 days
20 " " " 73 "	8 " " " 121 "
17 " " " 85 "	7 " " " 122 "
15 " " " 89 "	6 " " " 124 "
14 " " " 93 "	5 " " " 131 "
13 " " " 106 "	4 " " " 133 "
12 " " " 110 "	3 " " " 136 "
11 " " " 112 "	

The experiment terminated during a heat wave, when the laboratory temperature was over 70° F.

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XLV. THE APPARENT FORMATION OF EU- GLOBULIN FROM PSEUDO-GLOBULIN AND A SUGGESTION AS TO THE RELATIONSHIP BETWEEN THESE TWO PROTEINS IN SERUM.

BY HARRIETTE CHICK.

(From the Lister Institute.)

(Received July 15th, 1914.)

INTRODUCTION.

A great difficulty in completely separating water-insoluble protein has been the common experience of all who have attempted to prepare pure salt-free samples of pseudo-globulin. In the course of fractionating horse-serum for viscosity measurements [Chick 1914], I found that, however carefully the euglobulin of the serum had been previously separated, the final product invariably contained more or less water-insoluble protein. Haslam [1913] devoted much attention to this question and came to the conclusion that the presence of the latter was due to incomplete removal of the euglobulin in the original serum. This is to some extent true, but my experience has led me to believe that the bulk of the water-insoluble material cannot be accounted for in this manner, for a protein, having the characteristics of euglobulin, was found to increase progressively in amount as the purification of the pseudo-globulin proceeded. A small amount of water-insoluble protein can usually be detected immediately after the first separation of the pseudo-globulin by salt precipitation and this does appear to consist of euglobulin originally present in the serum. The comparatively large quantity (in one case, Exp. IV, Table III, nearly one-fifth of the total protein) present at the end of a lengthy dialysis appears, on the other hand, to be derived, at least in part, from a transformation of the pseudo-globulin itself. The experiments about to be described show that, under certain circumstances, pseudo-

globulin may undergo change into a protein possessing the properties of euglobulin and suggest that such may very likely be the origin of the euglobulin present in serum.

ORIGIN OF THE WATER-INSOLUBLE PROTEIN FOUND PRESENT IN
PSEUDO-GLOBULIN.

In one set of experiments (Table I) pseudo-globulin was prepared from diluted horse-serum by the following method, rapidly executed. Ammonium sulphate was first added to one-third¹ saturation, the precipitate separated and the filtrate brought to half saturation of ammonium sulphate, this second precipitate being pressed free from mother liquor and afterwards dissolved in a small amount of water. The presence of a small amount of insoluble protein was always apparent on dilution, especially if the solution were adjusted to a definite, slightly acid, reaction.

This insoluble protein we may regard as euglobulin not thrown out from serum by one precipitation in one-third saturated ammonium sulphate for if, in addition to this process, as much as possible of the euglobulin is previously removed by careful acidification of the diluted serum, the water-insoluble protein which can be detected in the precipitated pseudo-globulin is considerably reduced (see Table II).

When pseudo-globulin (horse), after undergoing one or more re-precipitations, is subjected to a lengthy dialysis to remove ammonium sulphate, it is found to contain a comparatively large amount of water-insoluble protein. As a general rule from one-tenth to one-fifth of the total protein is found to be in this condition. This insoluble material exhibits a striking likeness to euglobulin. It is dispersed by small quantities of dilute acid or alkali and when first separated is dissolved by neutral salts, which latter property, as is the case with euglobulin, becomes impaired with increasing age of the preparation.

It has not been possible to determine with certainty the origin of the whole of this "euglobulin." One view is that it is derived from the pseudo-globulin by some process of "denaturation"² or degradation taking place

¹ "One-third" and "one-half" saturation indicate the concentrations of ammonium sulphate obtained when a saturated solution of ammonium sulphate is added to twice its volume or an equal volume of water.

² "Denaturation" of proteins cannot at present be identified with any chemical changes in the material. The term "denaturated" (German, *denaturiert*) is applied to proteins possessing a certain group of solubility characteristics, including a total insolubility in water at the iso-electric point, in absence of electrolytes. Solution (dispersion) in water occurs in the presence of acid, alkali or electrolytes, and is associated with the acquisition of an electric charge by the protein particles, while the system displays a high degree of viscosity. The best-known example is that

after separation from the serum and the results of Exps. I-IV set forth in Tables I-III may, on the whole, be said to support this view. On the other hand, it may be urged that the results of these experiments do not necessarily prove that dialysis of salt-precipitated pseudo-globulin is accompanied by its "denaturation," but that removal of all salt may be essential to permit complete precipitation of any "denaturated" protein (euglobulin) present from the beginning. If this is the case, the concentration of euglobulin in serum must be far higher (two or three times) than is generally believed and the usual method of separation, by dilution and acidification, very inadequate.

In order to throw light upon this point, some experiments were made in order to ascertain with what degree of accuracy water-insoluble protein can be estimated in presence of salts and other soluble protein. In one experiment euglobulin was mixed with a sample of thoroughly dialysed pseudo-globulin (from which all insoluble protein had been separated) and ammonium sulphate added; the mixture contained 0.462 % euglobulin, 2.02 % pseudo-globulin and 1.2 % ammonium sulphate. The concentration of insoluble protein was then estimated by the dilution and acidification method described in detail below. The value obtained was 0.337 %, or 73 % of that added. The concentrations of protein and salt in the final acidified, and diluted, solution were 0.048 % and 0.021 % respectively.

A second experiment was made with a thoroughly dialysed pseudo-globulin solution containing 1.77 % total protein, of which about one-seventh was in the "denaturated" condition and present as a precipitate. Estimations of the insoluble protein present in 10 cc. of the above solution were made both before and after addition of 5 cc. of strong ammonium sulphate (10.3 %). In the control, dilution 50-fold, the proportion of insoluble protein was found to be 0.70 %, in the presence of ammonium sulphate the estimated concentration was only 0.23 % and 0.26 % when diluted 50-fold and 100-fold respectively. In this experiment the final concentration of protein and ammonium sulphate was 0.10 % and 0.093 % respectively in the 50-fold dilution and half these amounts in the 100-fold dilution. The proportion of the total insoluble protein which could be precipitated under these conditions was 33 % and 37 % in the two cases.

of proteins which have been acted upon by hot water [see Chick and Martin 1910, 1911 and 1912]. The vegetable globulins and the euglobulin naturally occurring in serum may be regarded as "denaturated" proteins [see Michaelis and Rona 1910, 2]; their property of forming electrically-neutral solutions with certain salts is, however, a special characteristic not shared by heat-"denaturated" proteins.

When whole horse-serum is diluted ten times, as is usual when euglobulin is separated from serum, the total concentration of protein is from 0.6 % to 0.8 % and that of sodium chloride from about 0.08 % to 0.07 %. Under these circumstances, it is quite possible for some euglobulin to escape precipitation when the diluted serum is adjusted to the iso-electric point of euglobulin by the addition of acetic acid. On the other hand, it must be remembered that a chloride is a less efficient solvent for euglobulin than a sulphate [see Mellanby 1905]. Further, there is, as a rule, no suggestion of incompleteness about the process. The euglobulin usually settles with comparative ease, leaving a clear liquid on top.

The results of Exp. III, Table II also throw some light upon this question. In this case a solution of pseudo-globulin made immediately after its precipitation from the horse-serum contained 5.2 % ammonium sulphate, 12.86 % total protein, and only a trace of water-insoluble protein, when subjected to analysis by the method described below. Part of the material was kept in the laboratory as a control and part was dialysed, toluene being present in both cases to prevent decomposition. At the end of two months the water-insoluble material in the control was equal to 8.6 % of the total protein. In the portion which had been dialysed for 51 days the proportion estimated was 14.1 %. This experiment shows that water-insoluble protein, if present, can be detected in presence of electrolytes and other soluble protein. Therefore, a large proportion, at least 61 % (8.6 parts out of a total of 14.1) of the euglobulin found present at the close of dialysis must be regarded as formed from the pseudo-globulin by some process of denaturation taking place after separation from the serum. The remaining 39 % may, on the other hand, be regarded as euglobulin which was present from the first, although it could not be separated in presence of salt. But, from the fact that immediately after preparation only a trace of insoluble protein could be detected in the salt-containing pseudo-globulin, it seems probable that part of this may also be derived from the pseudo-globulin, showing that the denaturation of this protein is accelerated by dialysis.

EXPERIMENTAL METHODS.

In the experiments set forth in Tables I to III pseudo-globulin was prepared in various ways by salt-precipitation from horse-serum, the details of the method employed being given at the top of each table. Estimations of the denaturated protein were made before, after, and, in some cases

(Experiments I and II, Table I), during the dialysis, by determining the amount of protein which was precipitated by dilution and adjustment to the iso-electric point.

The method of estimation was as follows. A small portion of the material was taken for a preliminary trial, and largely diluted, so that the protein-content did not exceed 0.1 or 0.2 %. If electrolytes are present the dilution should be as large as possible. 10 cc. of this diluted solution were then placed in each of a series of test tubes and various small quantities of *N*/100 acetic acid added. If any denaturated protein were present an opalescence or slight turbidity was usually apparent on mere dilution and, when the solution was adjusted to the iso-electric point of the protein by addition of the required amount of acid, a precipitate was formed which aggregated and settled. The series of trial test tubes was always allowed to stand several hours and the composition of the material in the tube in which the precipitate was largest and agglutination most perfect was taken as the pattern for the estimation.

5, 10, or 15 cc. (according to the protein-content) were taken for analysis, diluted suitably and addition made of dilute (*N*/10 or *N*/100) acetic acid in the amount necessary for complete precipitation of the denaturated protein as calculated from the results of the preliminary trial. The whole was allowed to stand 24 hours and then centrifuged, the deposit being transferred to a small, weighed, centrifuge tube, spun down again, washed with distilled water slightly acidified and centrifuged a third time. The final deposit was dried in the weighed tube at 105° until constant in weight.

It is not advisable to permit the estimation to extend over more than one day, as, in some cases, the denaturation of the pseudo-globulin seems to be continued at an appreciable rate in the dilute solution and, when several days are spent over the estimation, higher values are frequently obtained.

If the material to be analysed is slightly acid, small quantities of dilute ammonia must be substituted for the dilute acetic acid. In cases where the material has been dialysed for a long time and the reaction is approaching that of the iso-electric point, dilution with distilled water containing about one-thirtieth of its volume of water saturated with carbon dioxide is often found to be a quick and efficient method of precipitating all the denaturated protein.

If electrolytes are present, denaturated protein may be detected by the above method, but precipitation, under certain circumstances, is incomplete (see above p. 405). In any case it is advisable to make the dilution as great as is possible.

The above method has been described in detail, because the estimation of denaturated protein in presence of other, water-soluble, protein is by no means an easy matter even in the absence of electrolytes, the difficulty being to ensure complete precipitation of the former. When this has not been accomplished, faint turbidity can be detected in the supernatant fluid after centrifuging; in a successful estimation the aggregation of the precipitate should be complete and the top liquor clear. Some of the earlier values for concentration of "denaturated" proteins given in Tables I to III were obtained when the method was not perfected and may be a little inaccurate; the later ones were more satisfactory, as may be seen in some cases where the results of duplicate analysis are inserted.

It was possible that this water-insoluble protein, like euglobulin [Freund and Joachim 1902, Gibson 1906, Banzhaf 1911], might be thrown out by addition of sodium chloride to saturation, and this would have proved a convenient method of separation and estimation. It was tried in Exp. II,

Table I, but without success. After diluting the material 10-fold and saturating with sodium chloride the residual protein (calculated on the original, undiluted, material) was 10.78 %, the total being 11.12 %; that is to say only 0.34 % protein had been thrown down. The concentration of "denatured" protein as separated by the above method was, however, found to be 0.96 %.

Precipitation by saturated sodium chloride is of little value for the accurate separation of proteins, the amount precipitated depending, to a greater degree than is the case with other salts, upon external conditions, such as the total concentration of protein and the reaction of the material. For example, on one occasion, with horse-serum, the precipitate obtained on saturation with sodium chloride was four times greater when the serum was diluted 1 in 10 than when the dilution was only 1 in 2; in the latter case the higher concentration of total protein protected more than three-quarters of the otherwise precipitable protein. Further, if diluted serum is made slightly acid in reaction, the whole of the pseudo-globulin and albumin is precipitated as well as the euglobulin.

DISCUSSION OF RESULTS.

The results of the experiments in Tables I-III suggest that the pseudo-globulin undergoes a progressive denaturation after its separation from the serum. For example, in Exp. I, Table I, the pseudo-globulin immediately after precipitation from the serum showed only a small amount of water-insoluble protein capable of separation by dilution and adjustment of the reaction to the iso-electric point. At the end of dialysis for 10 and 18 days, however, 9.1 % and 12.9 % respectively, of the total protein was found to be in the insoluble condition. It is probable that the ammonium sulphate present interfered with a complete separation of any euglobulin present at the beginning of the experiment, but it is unlikely that the difference in amount of the traces of salt present after 10 and 18 days' dialysis respectively could be responsible for the large difference in amount of insoluble protein found on these two occasions.

Experiment IV, Table III is a second instance. Here material dialysed for five days against tap water contained 11.1 % denatured protein: after a further dialysis against distilled water lasting 29 days this proportion was increased to 18.1 %.

TABLE I.

Spontaneous formation of euglobulin from pseudo-globulin at ordinary temperature; pseudo-globulin prepared by half-saturation of undiluted horse-serum with ammonium sulphate, after separation of euglobulin by one-third saturation with ammonium sulphate.

Exp.	Material	Date of analysis	Length of dialysis, days	Total protein, %	Water-insoluble protein, %	G. water-insoluble protein per 100 g. total protein	Phosphorus-content, g. P per 100 g. protein	
							in water-soluble fraction	in water-insoluble fraction
I.	Prepared 2. 12. 13; re-precipitated twice; final precipitate dissolved in water. Dialysed in presence of toluene against distilled water, changed daily, from 5. 12. 13 to 23. 12. 13	5. 12. 13			small amount			
		15. 12. 13	10	14.56	1.33	9.11		
		14. 3. 14 ¹	18	13.55	1.75	12.9	0.018	0.171
II.	As in Exp. I but not re-precipitated	6. 12. 13	3	11.17	present			
		26. 1. 14 ²	3	11.12	0.964	8.67		
	(a) dialysed against dist. water from 13. 2. 14 to 25. 3. 14	3. 4. 14 ³	43	4.77	0.654	13.7		
		9. 7. 14	43	4.77	0.700	14.7		
	(b) as above, but dialysed against dist. water saturated with CO ₂ from 13. 2. 14 to 25. 3. 14	26. 3. 14	43	5.51	{ 0.652 } ⁴ (0.660)	11.9		

¹ Kept in cold room from 23. 12. 13 until 14. 3. 14.

² Kept in cold room from 6. 12. 13 until 13. 2. 14; dialysis then recommenced.

³ Kept in cold room from 25. 3. 14 until 9. 7. 14.

⁴ Duplicate analyses.

TABLE II.

Spontaneous formation of euglobulin from pseudo-globulin at ordinary temperature; pseudo-globulin prepared from diluted (1 in 20) horse-serum by half-saturation with ammonium sulphate, after successive removal of euglobulin precipitates obtained (1) by acidification of the serum (1 in 10 dilution), and (2) by one-third saturation of the serum (1 in 15 dilution) with ammonium sulphate: once re-precipitated and dissolved in a small quantity of water.

Exp.	Material	Date of analysis	Length of dialysis, days	Concentration of $(\text{NH}_4)_2\text{SO}_4$, %	Total protein, %	Water-insoluble protein, %	G. water-insoluble protein per 100 g. total protein	Phosphorus-content g. P per 100 g. protein	
								in water-soluble fraction	in water-insoluble fraction
III.	Control, immediately after preparation	4. 2. 14	—	5.23	12.86	trace	—	—	—
	(a) Control, allowed to remain at laboratory temperature, in presence of toluene	3. 4. 14	—	5.23	2.86	1.107	8.61	—	—
	(b) Dialysed against distilled water, changed daily, in presence of toluene from 2. 2. 14 to 25. 3. 14	30. 3. 14	51	—	4.86	$\left\{ \begin{array}{l} 0.688, \\ 0.681 \end{array} \right.$ ¹	14.1	0.012	0.262

¹ Duplicate analyses.

TABLE III.

Spontaneous formation of euglobulin from pseudo-globulin at ordinary temperature; pseudo-globulin prepared from horse-serum (diluted 1 in 4) by precipitation with one-half saturated ammonium sulphate, this first precipitate extracted with saturated brine, brine-extract precipitated by addition of acetic acid to a concentration of 0.25 %¹; this second precipitate drained, pressed and mixed with washing soda to a concentration of 3 % and dialysed for five days against tap water.

Exp.	Material	Date of analysis	Length of dialysis, days	Total protein, %	Water-insoluble protein, %	G. water-insoluble protein per 100 g. total protein	Phosphorus-content, g. P per 100 g. protein	
							in water-soluble fraction	in water-insoluble fraction
IV.	Control	3. 3. 14	(5) ²	11.72	1.301	11.10	—	—
	(a) Dialysed further against dist. water, changed daily, in presence of toluene from 6. 3. 14 to 4. 4. 14	4. 4. 14	29 (+5)	5.87	1.055	17.98	0.0062*	0.156
	(b) as (a) the distilled water being saturated with CO from 6. 3. 14 to 4. 4. 14	4. 4. 14	29 (+5)	6.40	1.161	18.14	0.0066*	0.098

¹ Gibson's [1906] process.² Against tap water.

* Approximate only (less than 1 cc. N/10 NaOH neutralised).

The results of Experiments I to IV also suggest in every case that the degradation of the pseudo-globulin is approaching a limit. For example, in Exp. II, Table I, after three days' dialysis 8.7 % of the protein was found to be water-insoluble. After an interval of more than two months, including a second dialysis lasting 40 days, the proportion had only risen to 13.7 % and, after having been kept in the refrigerator for a further period of over three months, to 14.7 %. A similar result is shown in Exp. I. In many cases, the experiments were continued beyond the date of the last analysis given in the tables, and the denaturation process was found to be practically at an end. The degraded material was separated as completely as possible by a modification of the method described above, and the clear, top liquor adjusted to the iso-electric point of the denaturated protein and kept for a considerable time in the cold room under observation. In the case of Exps. III and IV (*a* and *b*), only a trace of insoluble material was deposited after periods of two months and one month respectively.

PHOSPHORUS-CONTENT OF THIS WATER-INSOLUBLE PSEUDO-GLOBULIN.

The resemblance between the denaturated pseudo-globulin obtained in these experiments and euglobulin led me to make estimations of the phosphorus contained respectively in the insoluble and unchanged pseudo-globulin.

Hardy [1905] demonstrated the presence of phosphorus (0.07 to 0.08 %) in euglobulin (ox-serum) and considered it to be one of the characteristics marking off this protein as a chemical entity. The pseudo-globulin (separated from the serum by saturation with magnesium sulphate) he found to contain a trace only (about 0.009 %) of phosphorus. Haslam [1913] also found phosphorus to be a constant constituent of euglobulin (ox-serum), even after repeated purification, and to be absent from the purest samples of pseudo-globulin which he was able to obtain. In consequence, he used the absence of phosphorus as a criterion of the purity of pseudo-globulin.

In the present case phosphorus was determined by Neumann's method, using N/10 alkali and acid for the final titration, the quantity to be estimated being very small. In some cases where the ammonium molybdate precipitate was only just visible and the total amount of standard sodium hydrate neutralised less than 1 cc., the values obtained must be regarded as approximate only; such values are indicated in the tables by an asterisk.

The results of the phosphorus estimations showed the resemblance between

the denaturated pseudo-globulin and euglobulin to be maintained in this respect also. The former was found to remove almost the whole of the small amount of phosphorus contained in the original pseudo-globulin preparation. In Exp. I, Table I, the water-insoluble protein at the close of the experiment contained nearly 10 times, in Exp. III, Table II, more than 20 times, as much phosphorus as the unchanged pseudo-globulin; in both cases that present in the latter was reduced to a mere trace. In Exp. IV the contrast was even greater; the denaturated pseudo-globulin contained 0.16 % phosphorus, while that remaining with the soluble protein was too small in amount to be estimated with any degree of accuracy.

Two different explanations might be advanced to resume these facts. According to the first, the process described in the last paragraph is merely the separation of the last traces of phosphorus-containing euglobulin derived from the original serum and present from the beginning. On this theory the cessation of the gradual precipitation of insoluble protein would naturally coincide with the disappearance of the phosphorus originally present in the solution. On the other hand, we may regard this precipitate containing protein and phosphorus, which gradually makes its appearance, as the product of a gradual "denaturation" of the pseudo-globulin which requires the co-operation of some phosphorus-containing body (probably a serum-lipoid) which is present in small quantity in the preparations employed. When this serum-lipoid is exhausted, the process is at an end.

The second explanation would appear to be the correct one for the following reason. After the removal of phosphorus from the pseudo-globulin solution, by the separation of the insoluble protein precipitate, addition of a weak emulsion of lecithin, in presence of salt, causes additional formation of a protein resembling euglobulin which can be precipitated on subsequent dialysis.

Handovsky and Wagner [1911] showed that, when emulsions of lecithin, and other lipoids extracted from serum, were added to dialysed serum, a precipitation of the protein took place, which was prevented if salts were present. I have been able to confirm these observations. Addition of a dilute lecithin emulsion produces only slight turbidity when added to solutions of pure egg- or serum-albumin, a fact also noticed by Handovsky and Wagner. In the case of a dialysed pseudo-globulin, an immediate precipitation of protein takes place, which is prevented if a small concentration of salt (1 % sodium chloride), acid, or alkali is present.

I have prepared artificial euglobulin from pseudo-globulin in the following

manner. A small amount of a watery emulsion of lecithin¹ was added to the salt-free material obtained at the close of Exps. III and IV, in which the denaturation process had ceased and from which the insoluble protein had been separated. An immediate precipitate occurred, which was prevented by the presence of alkali in minute proportion or of salt (NaCl), to a concentration of about 1 %. On subjecting the solution to dialysis for 14 days precipitation occurred of a protein, containing phosphorus, and with the characteristics of euglobulin.

SUGGESTIONS AS TO THE ORIGIN OF EUGLOBULIN IN SERUM.

On consideration of the results of the foregoing experiments it seems not improbable that euglobulin in serum is a complex material, formed from pseudo-globulin by association with some serum-lipoid, to the presence of which it owes its phosphorus-content.

Hardy [1905], on the other hand, has regarded phosphorus as an integral part of the euglobulin molecule. He [1905, p. 331] was unable to remove all the phosphorus from heat-coagulated euglobulin (ox-serum) by treatment with strong acetic acid and subsequent extraction with alcohol and ether. He therefore concluded that it could not be "due to entangled lecithin." Haslam [1913, p. 514] found that extraction of euglobulin (ox-serum) with boiling alcohol removed a yellow, fatty substance which contained phosphorus, the proportion of the latter remaining in the euglobulin being reduced to about one-half the original. It is, however, quite possible that complete extraction with alcohol or ether is rendered very difficult by the state of aggregation of the protein and that if fresh surfaces could in succession be exposed to the action of these solvents a larger proportion of phosphorus could be removed.

In this connection it is worthy of note that the available analyses of euglobulin, some of which are collected in Table IV, show no approach to constancy in the proportion of phosphorus present. Hardy found 0.07 to 0.08 % phosphorus in euglobulin prepared from ox-serum by dilution and acidification; Haslam found 0.108 % and 0.105 % respectively present in two different samples. In three specimens prepared by the same method from three separate samples of horse-serum, I found 0.12 %, 0.032 %, and 0.065 % phosphorus respectively.

¹ For this preparation I am indebted to the kindness of Dr H. Maclean. The lecithin was prepared by his own method [1914] from heart muscle.

TABLE IV.

Phosphorus-content of euglobulin.

No.	Description of material and mode of preparation from serum	Method	G. phosphorus per 100 grms. protein		Authority
			mean		
1.	Euglobulin (ox-serum) by dilution and acidification	Neumann	0.11 0.11	0.11	Haslam
2.	Euglobulin (ox-serum) by dilution and acidification	Carius	0.07 0.08	0.075	Hardy
3.	Euglobulin (horse-serum A) by dilution and acidification once re-precipitated and washed	Neumann	0.122 0.117	0.119	Chick
4.	Euglobulin (horse-serum A) after three subsequent re-precipitations	Neumann	0.092 0.096	0.094	Chick
5.	Euglobulin (horse-serum A) as (4), but after extraction of the euglobulin suspension with acetone followed by dry ether	Neumann	0.019* 0.020	0.020	Chick
6.	Euglobulin (horse-serum B) by dilution and acidification, twice re-precipitated	Neumann	0.033 0.032	0.032	Chick
7.	Euglobulin (horse-serum C) by dilution and acidification, four times reprecipitated	Neumann	0.069 0.062	0.065	Chick

* Less than 1 cc. of N/10 NaOH neutralised.

TABLE V.

Proportion of phosphorus contained in a sample of whole horse-serum and in the various proteins after separation by different methods.

No.	Material	G. P. per 100 g. protein	Mean
1.	Horse-serum; whole serum dried in vacuo at 37°	0.098 0.103	0.100
2.	Total proteins of horse-serum, precipitated by dropping into a large volume of absolute alcohol; washed with alcohol and ether; and dried at 105°	0.027 0.018*	0.027
3.	Total proteins coagulated in hot water, coagulum washed with water, alcohol and ether; dried at 105°	0.004* 0.008*	0.006*
4.	Euglobulin prepared from No. 6 by one-third saturation with ammonium sulphate (re-precipitated six times)	0.071	0.071
5.	Euglobulin prepared by dilution and acidification of the original serum (re-precipitated four times and washed twice with distilled water)	0.069 0.062	0.065
6.	Total globulin, prepared by one-half saturation with ammonium sulphate (re-precipitated six times)	0.023	0.023
7.	Serum albumin precipitated in the filtrate from No. 6 by saturation with ammonium sulphate (re-precipitated five times)	0.032	0.032

* Approximate only, less than 1 cc. N/10 NaOH neutralised.

A further set of analyses, see Table V, was made of the total globulin, and euglobulin of horse-serum, as well as the total protein precipitated by various methods. The specimens used were all prepared by Dr P. Hartley with great precautions and carefully purified and I am greatly indebted to him for generously placing this valuable material at my disposal. The results showed that none was free from phosphorus. In some cases the proportion contained showed great variation according to the method employed in preparation (*e.g.* nos. 1, 2, and 3, Table V). The euglobulin contained the greatest amount of phosphorus, and the content was about the same whether precipitated with ammonium sulphate or by dilution and acidification of the serum. This would indicate that the phosphorus contained in euglobulin was in closer association than the traces found present in the purified samples of the other proteins.

Any direct evidence regarding the character of the union of phosphorus in euglobulin is at present scanty, but the following results are worthy of consideration. Haslam [1913] was able to remove about half the total phosphorus from a sample of euglobulin by means of extraction with alcohol and ether. Absolute alcohol must, however, in case of proteins be regarded as a powerful reagent, causing serious changes; all are rendered permanently insoluble if contact is long enough and the temperature is allowed to rise above a low maximum. If previous to extraction with ether, acetone¹, in place of alcohol, is employed to remove the water, no damage appears to be suffered.

The material can then be extracted with ether, three or four changes being employed to remove completely all acetone, and the euglobulin remaining in contact with ether at room temperature for one or two days. The last ether extract being removed, the euglobulin is shaken up once more with ether, which is evaporated by pouring the euglobulin suspension on to a warmed porcelain basin and allowing a gentle current of warm air to play over it, the whole operation taking place in a hot room (about 36°). The extracted euglobulin is then obtained as a fine, white powder, which is exceedingly hygroscopic. The solvents employed should all be carefully purified and freed from water.

The extracted euglobulin was found to have lost nearly all its phosphorus; on one occasion it was reduced to less than one-quarter of the original (see Table V, original phosphorus-content 0.094 %; final 0.02 %). It is possible that the care taken to free all materials from water may account for the

¹ Acetone can be employed to remove water from the proteins of whole serum, without impairing their solubility in water.

large proportion of the phosphorus removed from the euglobulin in these experiments when compared with those of Haslam.

The ether-extracted euglobulin, like that treated by acetone, was readily soluble in water containing a small amount of acid or alkali. Its solubility in dilute salt solution was, however, always found to be reduced to a greater or less extent. On one occasion about 13 % was found to be soluble in dilute sodium chloride (0.85 %); in most cases the ether-extracted material was totally insoluble in salt solution.

The acetone and ether extracts, when taken to dryness, were found to contain fatty substances, presumably the lipoids contained in the original euglobulin. These fatty materials, which contained a considerable proportion of phosphorus, amounted in one instance to as much as 13.3 % by weight of the protein taken for the experiment. On one occasion, when the extracted euglobulin was rubbed up in a mortar with the fatty residue and salt solution, the power of salt solubility was found to have been restored to some extent, and a similar result was obtained with petrol-extracted euglobulin and the residue from the petrol extract. These two experiments were made with the same sample of euglobulin. With a second sample of euglobulin the experiment was unsuccessful and with a third a trace only was rendered soluble in dilute salt solution by this means. In the case of the first, successful, experiment a large proportion of the lipid extracted from a comparatively large quantity of euglobulin was devoted to a small fraction of the treated protein and this procedure appears to be necessary to obtain any union and consequent restoration of salt solubility.

Some experiments with ether-extracted euglobulin were also made in which a lecithin emulsion was substituted for the fatty substance extracted from the euglobulin itself. In some instances the lecithin-treated material had distinctly greater solubility in sodium chloride, in others little or no difference from the control could be demonstrated. The question is still under investigation.

The evidence contained in the preceding paragraphs in favour of regarding euglobulin as a mechanical complex consisting of a protein (pseudo-globulin) and a lipid may briefly be summarised as follows. The phosphorus-content is quite inconstant and shows wide variation in specimens prepared from different samples of serum. By extraction with acetone and ether, under certain circumstances, almost all the phosphorus can be removed together with a small amount of a fatty body. By this procedure the euglobulin, otherwise unchanged, loses its property of salt solubility, which characteristic

has, in some cases, been restored by addition of a lipid to the ether-extracted material.

As regards the nature of this complex, euglobulin in serum must, in my opinion, be regarded as the product of the interaction of two colloidal systems, viz. the colloidal solution of pseudo-globulin and the emulsion of some lipid present in the serum. The process is not reversible. The serum-lipoid remains firmly associated with the protein, and cannot be separated by repeated dispersion and re-precipitation, but can be removed by extraction with acetone followed by ether. The freed protein is in the "denaturated" condition, the pseudo-globulin having apparently undergone this irreversible change in the first instance under the influence of the lipid.

Walpole [1914] has pointed out that, in some respects, euglobulin shows analogies with certain artificial colloidal systems which he has investigated, as, for example, solutions of gelatin to which suspensions of mastic or emulsions of oil have been added. In these mixtures of two colloidal constituents with widely separated iso-electric points, the dual nature of the system is apparent in the want of accurate coincidence between the iso-electric point and that of optimum flocculation. The same phenomenon has been demonstrated in the case of euglobulin [Chick 1913] and may doubtless be similarly explained. Feinschmidt [1912] determined the iso-electric point of six samples of lipid from various sources to lie at a concentration of hydrogen-ions between about 10^{-2} and $10^{-6.7}$ normal. The iso-electric point of the protein, on the other hand, is at a concentration of hydrogen-ions somewhere between 10^{-5} and 10^{-6} normal. [Michaelis and Rona 1910, 1, Michaelis and Davidsohn 1911, Sørensen and Jürgensen 1911, Chick and Martin 1912.]

The identification of the protein in euglobulin with pseudo-globulin is justified on chemical grounds by some recent work of Hartley¹. He has shown that on examination of the three proteins (euglobulin, pseudo-globulin and albumin) of serum (horse and ox) by van Slyke's method, the composition of pseudo-globulin and euglobulin is found to be practically the same while a marked difference exists in case of serum albumin.

SUMMARY.

1. Pseudo-globulin, prepared from diluted serum by precipitation with ammonium sulphate, the euglobulin having been previously removed as far as possible by dilution and bringing the solution to the iso-electric point,

¹ Communicated to the Physiological Society, Feb. 14, 1914 (Lister Institute, London) and to the Biochemical Society, July 11, 1914 (Oxford).

was always found, at the end of a long dialysis, to contain large quantities of a water-insoluble protein, resembling euglobulin.

2. Evidence is adduced to show that this insoluble material is not merely euglobulin present in the original serum and precipitated on dialysis, but is largely derived from the pseudo-globulin by a gradual process of "denaturation."

3. The gradual precipitation of the insoluble protein is accompanied by a disappearance from the solution of the small amount of phosphorus originally present in the pseudo-globulin preparation, after which there is no further separation of water-insoluble protein.

4. On addition of a watery emulsion of lecithin to thoroughly dialysed pseudo-globulin, from which insoluble protein and phosphorus had been removed as above, the "denaturation" process could be re-initiated.

5. The presence of minute amounts of acid and alkali or of a small concentration of salt (e.g. 1 % sodium chloride) prevents the precipitation of pseudo-globulin by a watery lecithin emulsion but, on dialysis, an insoluble protein, containing phosphorus and displaying the properties of euglobulin, is separated.

6. This artificial euglobulin appears to be a mechanical complex resulting from the interaction and mutual precipitation of the two colloidal systems : (a) the solution of pseudo-globulin and (b) the lipid emulsion.

7. It is suggested that the euglobulin in serum is a protein-lipoid complex of similar origin. In support of this view the following facts are brought forward :

(a) The inconstant phosphorus-content.

(b) The readiness with which phosphorus can be removed from euglobulin by extraction with acetone and ether, during which process the property of salt solubility is lost. This can, in some cases, be restored by addition of the extracted fatty substance or of lecithin to the extracted euglobulin.

(c) The want of accurate coincidence between the iso-electric point of euglobulin and the point of optimum flocculation, as pointed out by Walpole [1914].

(d) The similarity in chemical composition between pseudo-globulin and euglobulin, as shown by Hartley.

In conclusion I desire to express my indebtedness to Prof. C. J. Martin, F.R.S. for valuable criticism.

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XL. THE RELATIONS OF VITAMINE TO LIPOIDS.

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Although vitamine, i.e. the substance which prevents beri-beri and avian polyneuritis, has been shown to be present in higher concentration in various tissues rich in lipid, e.g. liver, cardiac muscle, brain (ox) and egg-yolk, than in voluntary muscle (ox, fish) which is poor in lipid [Cooper, 1913, 1] it is now generally believed that the active substance as isolated from foodstuffs is not a lipid. It contains no phosphorus or carbohydrate [Cooper and Funk, 1911; Funk, 1911, 1914; Edie, Evans, Moore, Simpson, Webster, 1912] and is therefore neither a phosphatide nor cerebroside. MacLean [1912] furthermore showed that lecithin, which had previously been found by Schaumann [1910] to be slightly curative, had still less effect after purification, and he concluded that the anti-neuritic substance is not a lipid but is present in lipoids as an impurity extractable by simple methods. Cooper [1913, 2] subsequently found that when the fats and lipoids (alcoholic extract) of horse voluntary muscle were treated with ether the bulk of the anti-neuritic substance contained therein was precipitated and by a complex fractionation of the active precipitate a substance was isolated, small doses of which readily cured polyneuritis in pigeons. 4 g. of the original alcoholic extract exerted a rapid curative action in neuritic pigeons, but after one extraction with ether as much as 12 g. of the fats and lipoids (ether-soluble fraction) had only a slight ameliorative effect. More than 66 per cent. of the total amount of active material present in the original extract could thus be removed by simple treatment with ether and was thus apparently not in chemical union with the lipoids. Similar results were obtained when the flesh was not air-dried at 30° before extraction with alcohol [Cooper, 1913, 1]. This showed that the anti-neuritic substance was not split off from a lipid complex during the process of air drying.

The above facts however do not exclude the possibility that, although

the anti neuritic substance is preformed in the diet, in the nervous system it in part becomes chemically combined with other substances to form a lipid.

Experiments on similar lines have therefore been carried out with cardiac muscle and brain, and the results obtained are set forth in the present communication. The work with heart muscle was undertaken primarily with the object of isolating a sufficient amount of the anti-neuritic substance for a study of its chemical nature, the cardiac muscle being substituted for voluntary muscle on account of its higher content of active material [Cooper, 1913, 1].

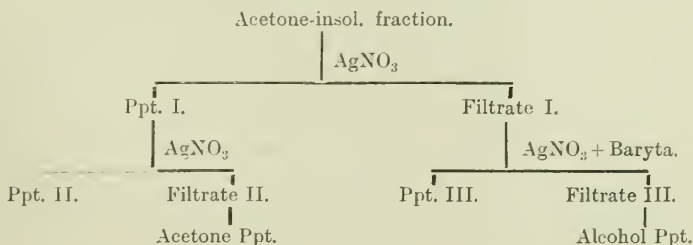
It must first be pointed out however that according to Vedder and Williams [1913] dry and wet beri-beri are caused by a deficiency in the dietary of distinct substances. In birds as a rule only dry beri-beri occurs, and, although infantile beri-beri is almost exclusively of the wet type, no signs of this condition were observed by the author even in very young chickens fed on polished rice. In this paper we are therefore only concerned with the substance which prevents and cures dry beri-beri and avian polyn neuritis.

I. PREPARATION OF AN ANTI-NEURITIC SUBSTANCE FROM CARDIAC MUSCLE.

150 lbs. of ox-heart were minced, dried at 30° by means of an electric fan, ground, and thoroughly extracted with 95 % alcohol. The filtered extract, from which the alcohol was removed by evaporation *in vacuo*, was treated with an excess of ether, and the material precipitated possessed marked anti-neuritic properties. 0.2 g. given orally rapidly curing pigeons affected with polyn neuritis. The procedure subsequently followed was a modification of that employed in the fractionation of horse-flesh and is briefly described below.

The ether-insoluble precipitate was taken up with 95 % alcohol which dissolved a considerable amount of material. The insoluble residue contained both inorganic and organic matter, but was inactive. The soluble fraction however was strongly curative and was next treated with excess of ether, which completely precipitated the anti-neuritic substance. The precipitate was dissolved in water and allowed to stand in a vacuum desiccator for several days. A white crystalline substance slowly separated, but this had no curative action. The supernatant liquor was then treated with basic lead acetate until no more precipitate was formed. As in the case of horse-flesh, the active substance was not precipitated by this reagent, and the filtrate

possessed marked curative properties. So far the method was identical with that employed in the case of horse-flesh, but at this stage a departure from the original process was made. It was found in the previous work that the addition of silver nitrate after the lead acetate precipitation only partially precipitated the curative substance. This manipulation was therefore postponed in the subsequent technique with the object of ascertaining what influence intermediate fractionations might have upon the precipitability of the substance by the silver salt. The lead acetate filtrate after the removal of excess of lead by dilute sulphuric acid was therefore evaporated *in vacuo* and the concentrated solution treated with excess of 95 per cent. alcohol. By this procedure some material was separated, but all the anti-neuritic substance remained in solution. The filtrate was evaporated to a syrup and treated with acetone, which precipitated practically all the active substance. The filtrate possessed only slight curative properties, whilst 0.15 g. of the precipitated substance given orally rapidly cured pigeons affected with polyneuritis. The substance was insoluble in benzene, ether, and chloroform, but dissolved readily in water. The aqueous solution was next treated with silver nitrate solution, which however still only precipitated a portion of the active substance, and the latter could furthermore only be partially precipitated from the filtrate by the addition of baryta. The first precipitate (with silver nitrate only) was decomposed with dilute hydrochloric acid and silver nitrate again added to the filtered solution. On this occasion the substance was not precipitated to any appreciable degree and the filtrate was strongly curative. The filtrate after removal of excess of silver as the chloride was concentrated *in vacuo* and was then treated with acetone. A substance was thereby precipitated, 0.04 g. of which given orally quickly cured polyneuritis in pigeons whilst 0.008 g. cured by subcutaneous injection. The filtrate from the third precipitate (obtained with silver nitrate and baryta) was concentrated and treated with 95 per cent. alcohol, and a substance was precipitated, 0.04 g. of which given orally also cured polyneuritis. The manipulations are set forth schematically below.



(The thick lines represent the main course of the active substance during the fractionations.)

The substances separated in the above way from filtrates II and III and the solution obtained by decomposition of precipitate III were now combined and treated with 95 per cent. alcohol, which precipitated a small amount of material possessing no curative properties. The filtrate was evaporated and acetone added. This caused the precipitation of a substance (5 g.) 0.04 g. of which administered orally readily cured neuritic pigeons. The filtrate was only slightly curative.

On standing a few days in a sulphuric acid desiccator the substance however ceased to have any curative action, and even large doses only prolonged the lives of neuritic birds. It was thus of little interest to carry out a chemical analysis of the substance.

It is evident therefore that the anti-neuritic substance is extremely labile and with the chemical methods at present at our disposal its isolation will prove a very difficult matter. Further work must be carried out to ascertain the conditions favourable to the existence of the active substance. For this reason and also on account of cost it is doubtful if highly purified preparations will be of much therapeutical value.

An inexpensive method however of preparing a non-toxic highly curative solution suitable for use in the treatment of human beri-beri was described in a previous communication [Cooper, 1914, 2].

II. THE RELATIONS OF VITAMINE TO LIPOIDS.

The curative power of the original alcoholic extract of cardiac muscle was determined before and after treatment with ether. It was found that about 3 g. of the extract readily cured polyneuritis in pigeons, but after the extract was treated with ether 4 to 8 g. of the fats and lipoids (ether-soluble fraction) had little or no curative action. The small amount of active substance still retained by them could not be removed by a second extraction with ether, but by treating the ether-soluble fraction with acetone the phosphatides were precipitated and as much as 10 g. had not the slightest effect upon neuritic pigeons. These observations support the conclusion of MacLean (p. 347).

Different observations however were made in the case of brain. 13 kilos. of ox-brain were minced, dried at 30° for 16 hours, and extracted with alcohol on the shaking machine. The filtered extract was concentrated

in vacuo to remove the alcohol, and when dry weighed 730 g. The extract was next treated with excess of ether, which precipitated 100 g. of a white substance. 0.6 g. of this rapidly cured polyneuritis in pigeons, but 0.1 g. had only a slight action.

This fraction from brain was less curative than the corresponding fractions from voluntary muscle and cardiac muscle, 0.2 g. of which was quite sufficient to bring about recovery. It also differed from the voluntary muscle preparations in not being completely soluble in water. The ether-soluble fraction from brain, on the other hand, unlike the analogous fractions from muscle, possessed marked curative properties, 2.3 g. being sufficient to cure pigeons affected with polyneuritis. The fats and lipoids still retained their activity even when extracted three times with ether.

On treating the ether-soluble fraction with acetone however the active substances were present in the acetone-soluble fraction, 1.3 g. of which readily cured, and, as was observed in the case of cardiac muscle, the precipitated phosphatides in doses of 5 to 10 g. possessed no detectable curative power. The phosphatides had no curative effect even after hydrolysis with 5 per cent. sulphuric acid, a process which does not affect the active substance. This shows that the negative results were not due to the indigestibility of the lipoids.

Similar results were obtained when alcoholic extracts of sheep-brain and egg-yolk, were treated with ether. There would thus appear to be present in brain and egg-yolk an ether- and acetone-soluble substance which possesses a marked affinity, chemical or adsorptive, for vitamine, and which may function as a carrier in the living organism.

The residues left after extracting the ox- and sheep-brain with alcohol were retained, and experiments carried out to ascertain to what extent the anti-neuritic substance contained in brain was extracted by alcohol.

13 kilos. of the ox-brain yielded 1680 g. of residue after exhaustion with alcohol. It was previously found [Cooper, 1914, 1] that the addition of 6 g. of ox-brain to the polished rice diet (1/20th body-weight ration daily) was sufficient to prevent polyneuritis in pigeons for at least 50 days.

Two pigeons were therefore fed daily on the above ration of polished rice and an amount of the brain residue equivalent to 12 g. of brain (1.5 g.). Both birds developed polyneuritis in 30 days. It is evident from this result that the greater part of the anti-neuritic substance contained in ox-brain is extracted by 95 per cent. alcohol.

1900 g. of the sheep-brain yielded 238 g. of alcohol-insoluble material.

It was previously observed [Cooper, 1913, 1] that the addition of about 8 g. of sheep-brain to a 1/20th body-weight ration of polished rice daily prevented polyneuritis in pigeons for 50 days. Three pigeons were therefore fed daily on the above ration of rice and an amount of the brain residue equivalent to 15 g. of the original brain (2 g.). All the birds developed polyneuritis within 30 days. As in the case of ox-brain, the bulk of the anti-neuritic substance is evidently extracted along with the fats and lipoids by 95 per cent. alcohol.

It having been demonstrated that brain phosphatides when purified possessed no curative power, experiments were next carried out to ascertain if the anti-neuritic substance were more firmly combined with certain other constituents of brain. Cholesterol prepared by crystallisation from an acetone extract of brain was first tested on neuritic pigeons, but 1 g. was found to exert no curative action.

1 kilo. of ox-brain was next minced and without drying repeatedly extracted with absolute alcohol at 40°. The extract was filtered and kept at 0° for 12 hours. By that time a yellowish precipitate (protagon) separated out. This was filtered off and washed with cold alcohol, the residue weighing 15 g. 2 to 5 g. of this deposit however had no effect at all upon neuritic pigeons.

5 g. were then treated with ether. The insoluble residue (3 g.) containing cerebroside (cerebrone) had no curative action, and kephalin (1 g.) precipitated from the ethereal extract by the addition of absolute alcohol also proved to be inactive. The original alcoholic extract after removal of the "protagon" was concentrated *in vacuo*, and the residue treated with ether. As before, the precipitate was curative. It was extracted with absolute alcohol at 0°, and the undissolved substance (1.5 g.) containing cerebrosides was again inactive. The alcohol-soluble fraction was concentrated *in vacuo* and when dry weighed 5 g. This was highly curative, 0.25 to 0.5 g. being adequate to cure polyneuritis.

The original ether-soluble fraction containing the phosphatides and fats of the brain was freed from ether and treated with acetone. The precipitated phosphatides were dissolved in a little chloroform and again reprecipitated with acetone. The phosphatides in doses of from 4 to 8 g. were inactive but 1 to 2.5 g. of the acetone-soluble fraction readily cured pigeons, thus confirming the observations detailed earlier in the paper.

This work was then repeated on a larger scale, 3 kilos. of ox-brain being used. Similar results were obtained, protagon (even when hydrolysed)

kephalin, cerebrone, and the phosphatides again being found to be ineffective when given to neuritic pigeons in approximately the above mentioned doses.

The failure of protagon, kephalin, cerebrone, cholesterol and phosphatides to cure polyneuritis is of interest in relation to the researches of Stepp [1909, 1911, 1913] who has found that food extracted with alcohol-ether is deleterious to the health of mice, and that the harmful effect of the diet is not checked by the addition of lecithin, cholesterol, kephalin, cerebrone, and phytin. When however egg-yolk or alcoholic extracts of egg-yolk or brain are added the animals remain healthy. Of these substances cholesterol, kephalin, and cerebrone as stated above do not contain vitamine, and lecithin [MacLean, 1912; Cooper, 1913, 2] and phytin [Schaumann, 1910; Cooper and Funk, 1911] when purified are also practically free from the active substance.

Egg-yolk and alcoholic extracts thereof on the other hand contain the essential substance [Cooper, 1913, 1], as also do alcoholic extracts of brain [Funk, 1912; Cooper, 1913, 2]. It is therefore probable that the harmful effects of diets consisting of food extracted with alcohol-ether is due not to the deficiency in the diets of lipoids, but to a deficiency of vitamine which is extracted by alcohol along with the lipoids.

The main conclusion that emerges from these observations is that vitamine does not apparently enter into the constitution of the lipoids of the brain, but may possibly be stored therein until required in the nutrition of the nervous system.

To what extent lipoids can adsorb the active substance from an *aqueous* medium is a point to be settled by further experiment. Meanwhile it may be noted that asbestos (Ural) does not appreciably adsorb vitamine from solutions of autolysed yeast (acid reaction).

SUMMARY.

1. Although infantile beri-beri is almost exclusively of the wet type, young chickens fed on polished rice develop symptoms of the dry form only, like adult birds.

2. A method is described for the preparation from cardiac muscle of a substance small amounts of which cure polyneuritis in pigeons. The substance however was extremely unstable and lost its activity in a few days after isolation.

3. As in the case of voluntary muscle, the bulk of the anti-neuritic

substance contained in the fats and lipoids (alcoholic extract) of cardiac muscle can be separated therefrom by means of ether.

4. In the case of brain, however, the anti-neuritic substance can only be partially precipitated from the alcoholic extract by treatment with ether, but can be entirely removed from the phosphatides by the subsequent addition of acetone. An explanation of this is offered.

5. Purified brain phosphatides possess no curative power even after hydrolysis with acid. This shows that their inability to cure polyneuritis is not due to incomplete digestion.

6. Protagon (even when hydrolysed), kephalin, cholesterol, and cerebrone similarly have no curative action.

7. The bulk of the anti-neuritic substance contained in sheep- and ox-brain can be extracted by 95 per cent. alcohol.

CONCLUSION.

The results show that vitamine does not enter into the constitution of the lipoids of brain and muscle, but may possibly be adsorbed in the lipoids and so stored, until required by the organism.

It would thus appear that the deleterious effect of lipid-free diets observed by Stepp is due not to the deficiency of lipoids, but to the mechanical removal of vitamine during the alcohol-ether extractions.

I desire to express my best thanks to Dr Hugh MacLean for kindly help in the course of this investigation.

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N^o 4

CCVI.—*The Volumetric Estimation of Carbon in Aliphatic Substances in the Wet Way.*

By EGERTON CHARLES GREY (Beit Memorial Research Fellow).

THE original method of estimating carbon in the wet way by heating the substance with potassium dichromate and sulphuric acid or with chromic acid, with which the names of Rogers, Brunner Ullgrens, and Classen are associated, is described by Fresenius ("Anleitung zur quantitativen Chemische Analyse"). The substance was at first heated with the chromic acid mixture in a stream of air, but later Classen introduced a reflux condenser, whereby the distillation of water was prevented, and also traces of volatile chromium compounds. The method has been chiefly employed in the analysis of soils (Wolff, "Anleitung zur chemischen Untersuchung landwirtschaftlich wichtiger Stoffe"), and for carbon in steel and iron (Blair, *J. Amer. Chem. Soc.*, 1896, **18**, 223). In the case of soils, however, the method gave low results (Warrington and Peak, T., 1880, **37**, 617, and Cameron and Breazeale, *J. Amer. Chem. Soc.*, 1903, **26**, 29), which have been variously attributed to the formation of carbon monoxide, acetaldehyde, acetic acid, and other substances not completely oxidised.

It has been most generally assumed that low results are due to

carbon monoxide, but although under certain conditions this gas may be formed, its production can certainly be avoided in the case of aliphatic substances by using a proper excess of oxidising agent. On the other hand, it is more likely that the low results observed by many workers were due to acetic acid, which substance is attacked only with great difficulty by the chromic acid mixture.

In the present communication a method is described for the estimation of the total carbon in aliphatic substances, which depends on the gasometric measurement of carbon dioxide, followed by the determination of the acetic acid in the residual fluid, and it is shown that the method gives accurate results.

The apparatus employed is very similar to that already described by Blair, but mercury is used in the place of the salt solution employed by that author. This form of apparatus, in which the solution of the substance is allowed to boil in contact with the oxidising agent until completely oxidised either to carbon dioxide or to a mixture of carbon dioxide and acetic acid, is clearly better in principle than the method in which the products are allowed to escape from the sphere of the reaction in a partly oxidised condition.

Messinger (*Ber.*, 1890, **23**, 2756) overcame the difficulty of incomplete oxidation by allowing the vapours from the chromic acid mixture to pass through a combustion tube containing red-hot copper oxide, and lead chromate and potassium dichromate have also been employed in the same way (Hall, Miller, and Marmu, *T.*, 1906, **89**, 595), but the introduction of these modifications deprives the method of all its original simplicity.

The author has devoted his attention to this method chiefly with the object of applying it to the estimation of substances of biological importance, such as the alcohols and amino-acids, for which accurate methods are not available. The estimation of acetic acid, as well as of carbon dioxide, makes the method of general application to all aliphatic substances which are likely to occur in biological fluids, and the method has a further valuable application in the accurate estimation of alcohol and other volatile substances in dilute solutions.

The method has a great advantage in that it throws light on the constitution of the substance at the same time as it determines the percentage of carbon, for the titre of the acetic acid indicates the number of methyl groups in the compound; thus ethyl alcohol is oxidised practically quantitatively to acetic acid; propionic acid, and its derivatives, lactic acid and alanine, yield acetic acid corresponding with two carbon atoms out of three, whereas rhamnose yields two atoms of carbon out of the six in the form of acetic

acid. In each case the methyl group, together with the next adjacent carbon atom, forms acetic acid.

Ethyl alcohol, $\text{CH}_3 \cdot \text{CH}_2 \cdot \text{OH} \rightarrow \text{CH}_3 \cdot \text{CO}_2\text{H}$.

Alanine, $\text{CH}_3 \cdot \text{CH}(\text{NH}_2) \cdot \text{CO}_2\text{H} \rightarrow \text{CH}_3 \cdot \text{CO}_2\text{H} + \text{CO}_2$.

Rhamnose (*isodulcitol*), $\text{CH}_3 \cdot \text{CH}(\text{OH}) \cdot [\text{CH} \cdot \text{OH}]_3 \cdot \text{CHO} \rightarrow$
 $\text{CH}_3 \cdot \text{CO}_2\text{H} + 4\text{CO}_2$.

A small amount of acetic acid is at the same time oxidised to carbon dioxide, so that the value for the latter is greater than would theoretically be the case if the whole of the methyl group, together with the next adjacent carbon atom, were converted into acetic acid. The excess of carbon dioxide formed in this way is greatest when the oxidising fluid contains least water.

In the place of chromic acid, which, it seems, is always used with phosphoric acid, potassium dichromate has been used in this work. It has many advantages over chromic acid apart from its being free from impurities, since it may be weighed out and introduced into the flask much more conveniently than chromic acid. Moreover, only by using potassium dichromate, and avoiding the dilution of the oxidising medium by water, has it been found possible to effect the complete oxidation of carbamide.

It should be noted that the weight of material used for the analysis in many cases is considerably less than that generally employed for a dry combustion, and the method is certainly readily adaptable to microchemical analysis.

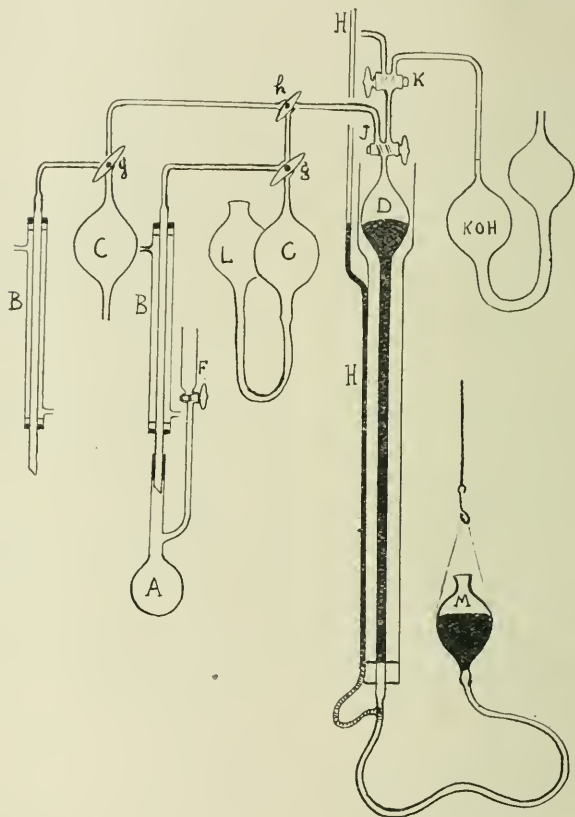
With most substances the oxidation is complete in from fifteen to thirty minutes, but others, such as carbamide and succinic acid, require longer, it being necessary to continue the heating for three hours. The operation requires, however, only a few minutes' actual attention, and two flasks with reflux condensers may be quite conveniently attached to the one gas analysis apparatus, so that two analyses may be carried out concurrently.

EXPERIMENTAL.

The apparatus employed is figured below. The gases are collected in the reservoirs rather than directly in the gas-measuring burette for several reasons. It will suffice to mention that in this way two flasks may be used in conjunction with the one burette, and, moreover, the burette itself is kept free from any traces of chromate which occasionally, although rarely, pass into the gas-collecting apparatus when the gases are swept out of the flask with the hot water.

Details of the Manipulation.—Fifteen grams of potassium dichromate are introduced into the flask A, which has a capacity

of about 75 c.c., and to this is added 15 c.c. of phosphoric acid (D 1.75). The flask is then attached to the reflux condenser *B* (internal tube 30×1 cm.), which is in communication through a capillary tube with the reservoir *C*. The tap of the funnel *F*, after having been smeared with phosphoric acid, is closed, and heat is applied to the flask *A*, the three-way tap *g* being turned so as to put *C* in communication with *B*, the vessel *L* being at the same



time lowered. About 4 c.c. of the fluid to be examined is now introduced into the funnel *F* (it is most convenient to weigh out the fluid from a bent pipette), and as soon as the potassium dichromate has mostly dissolved, and the chromic acid been set free, the solution is gradually allowed to be drawn into *A*. The funnel is then washed out into the flask with a few c.c. of water, followed by phosphoric acid, which is added to prevent the dilution of the oxidising fluid.

The heating is now continued for about thirty minutes, unless carbamide or succinic acid is present, when three hours is necessary. At the end of this time the flame is removed, the water allowed to run out of the condenser, and the residual gases in the flask are displaced by introducing hot water which has been boiled to remove carbon dioxide, into the flask *A*, through the funnel *F*, a lower pressure having been created within the flask by lowering the vessel *L*. The tap *g* is closed as soon as the hot water is seen in the capillary connecting *B* and *C*. The sample of gas may now be drawn into the measuring burette by lowering *M* and opening *J*. The adjustment to atmospheric pressure is made approximately by levelling with the mercury manometer tube *H*, and the fine adjustment is made by putting the gas into connexion with the potassium hydroxide of the Hempel bulbs. The measurement of the carbon dioxide is then carried out in the ordinary way.

When methyl groups may be present in the substance to be examined, the fluid, including that in the inner tube of the reflux condenser, is transferred when cold to a distilling apparatus, and distilled in steam in an atmosphere free from carbon dioxide.

The distillation in steam requires special precautions. The steam is generated by boiling distilled water treated with baryta, and to prevent minute traces of phosphoric acid being carried over into the distillate, the steam, after passing through the boiling acid solution, is made to pass through a second flask before being condensed. The lower tube of the condenser passes through a doubly bored rubber stopper into the neck of a large separating funnel, which acts as a receiver for the condensing fluid, and is protected from the access of atmospheric carbon dioxide by means of a soda-lime tube connected to a glass tube passing through the second hole of the rubber stopper. In this way, when fluid is withdrawn from the funnel, air free from carbon dioxide takes its place.

The results obtained with certain representative aliphatic substances are given below. Results with fatty acids of high molecular weight will be reserved for a later communication.

It must be mentioned that the phosphoric acid employed contained organic impurity, so that an allowance of from 1.5 to 2.6 c.c. of carbon dioxide had to be made in each experiment, according to the length of heating. Inasmuch as the amount of carbon dioxide produced from the phosphoric acid varies with the time of heating, it is of advantage previously to heat the phosphoric acid with a little chromic acid for some hours under a reflux condenser. A large amount of phosphoric acid may be readily

treated in this way, and its use is of advantage, especially when working with very small amounts of material.

Substance.	Weight taken.	Moist CO ₂ .	Temp.	Press.	Baryta N/30.	Carbon found.			Theory†
						As acetic acid.	As CO ₂ .	Total.	
Sucrose ...	0.0353	30.1	20.0	765	—	—	42.13	42.13	42.11
" ...	0.0745	65.2	26.0	769	—	—	42.17	42.17	
" ...	0.0657	56.8	23.5	771	—	—	42.35	42.35	
Glycerol ...	0.0992	79.65	23.5	763	—	—	38.86	38.86	39.13
" ...	0.0488	39.31	23.2	761	—	—	38.97	38.97	
Mannitol...	0.0425	33.00	15.0	762	—	—	39.16	39.16	39.56
Uric acid	0.0160	11.30	15.0	757	—	—	35.35	35.35	35.71
Carbamide	0.0782	31.96	21.6	764	—	—	20.01	20.01	20.00
Succinic acid.....	0.0191	15.70	22.5	767	—	—	40.23	40.23	40.66
Cystine ...	0.0270	15.18	24.0	761	—	—	30.30	30.30	30.00
Ethyl alcohol..	0.09638	2.3	23.3	754	61.27	50.87	1.16	52.03	52.17
Propionic acid.....	0.07424	36.0	28.0	765	23.31	23.02	25.13	48.15	48.65
Lactic acid	0.0714	25.53	26.0	766	20.40	17.37	22.85	40.22	40.00
Alanine ...	0.0733	18.54	23.0	761	22.82	12.26	27.78	40.04	40.44
"	0.1097	31.98	25.3	770	36.36	14.12	26.52	40.64	
Rhamnose	0.1170	66.95	27.0	769	17.32	27.43	11.84	39.27	39.55
"	0.1265	72.00	26.0	769	18.78	27.42	11.88	39.30	

General Conclusions.

Aliphatic substances can, in general, be oxidised to carbon dioxide, or to a mixture of carbon dioxide and acetic acid, by using potassium dichromate and phosphoric acid.

A volumetric method is described for the estimation of total carbon which is based on the estimation of these two products, the carbon dioxide being determined gasometrically and the acetic acid by titration.

The method described is particularly useful when the substance is already dissolved, and also in the case of volatile substances. A further advantage is that, at the same time, an indication is given as to the number of methyl groups in the chain.

Since, under the conditions of the experiment, ethyl alcohol is practically quantitatively oxidised to acetic acid, the method will be particularly of value in the determination of small concentrations of alcohol, where the error on the specific gravity method is great. Moreover, alcohol could be determined in the presence of any compounds which oxidise quantitatively to carbon dioxide, such as sugars and polyhydric alcohols, without the necessity of separation by distillation. In a concentration of 0.5 per cent., and

using only 4 c.c. of the fluid, a determination of alcohol may be made by this method with an error of less than 1 per cent.

The discussion of the applications of this method to the analysis of mixtures of two fatty acids or alcohols, as well as to the determination of formates, will be reserved for a subsequent communication.

In conclusion, I would express my thanks to Professor Arthur Harden, F.R.S., for help and criticism.

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N^o. 5

LVI. THE DETERMINATION OF THE COMPOSITION OF THE DIFFERENT PROTEINS OF OX AND HORSE SERUM BY THE METHOD OF VAN SLYKE.

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(*Received Sept. 12th, 1914.*)

Recent investigations have shown that serum albumin and serum globulin exhibit well marked differences in chemical composition. Abderhalden [1903, 1905] working with the proteins of horse serum found that albumin yielded no glycocoll on hydrolysis, while globulin gave 3·5 % of this amino acid. Haussmann [1899] and Gumbel [1904] studied the albumin of horse serum by Haussmann's nitrogen distribution method and Gibson [1912] using the same method determined the nitrogen distribution in the pseudo-globulin from horse serum. The results obtained by these three authors show that albumin contains a greater proportion of basic or diamino nitrogen than globulin. Bywaters and Tasker [1913] determined the sulphur and carbohydrate content of the albumin and globulin of horse serum. They found that albumin contains about 2 % of sulphur and 0·25 % of carbohydrate, while globulin yields less sulphur (1·17 %) and more carbohydrate (3·23 %). They also demonstrated the presence of phosphorus in globulin but could find none in albumin.

The globulin occurring in serum is of two kinds, viz. euglobulin and pseudo-globulin. The physical properties and solubilities of these two substances are very different, and recently evidence has been brought forward to show that they differ also in chemical composition. Hardy [1905] was the first to show that globulin contained phosphorus. He was unable to prepare a phosphorus-free globulin by extraction with alcohol and ether and concluded that the phosphorus of globulin is not due to entangled lecithin. Hardy also

succeeded in preparing an osazone from the insoluble phosphorus-containing globulin but failed to do so from the soluble phosphorus-free globulin. Haslam [1912, 1913] prepared pseudo-globulin free from phosphorus and showed that euglobulin contains about 0.1 % of phosphorus, about half of which he was able to extract by means of alcohol and ether. Hardy and Haslam worked with the proteins of ox serum.

In the experiments described in this paper the different proteins of ox and horse serum have been prepared and their chemical composition has been determined by the method of protein analysis recently introduced by van Slyke [1911]. This method has the advantages of yielding approximately quantitative results and requiring the use of relatively small quantities of material, and indicates the nature of the nitrogenous products resulting from complete acid hydrolysis. The whole proteins of the serum, the albumin, the total globulin, the pseudo-globulin and the euglobulin have been prepared in as pure a condition as possible. Each protein was finally obtained in the dry condition and weighed quantities of the air-dried preparations were analysed. Duplicate analyses were carried out in all cases in which sufficient material was available. Details of the preparation of the various proteins and the figures obtained in the analyses of the same are recorded at the end of the paper.

RESULTS.

The proteins of ox serum were studied first. Unfortunately, the amount of pure protein available was not sufficient in any case for duplicate analyses to be carried out, and in one case (*viz.* the euglobulin prepared from ox serum by one-third saturation with ammonium sulphate) only 2.5 g. of material were used. After hydrolysis the solution employed for analysis in this instance contained 235 milligrams of nitrogen: of this, only 52 milligrams were present as diamino nitrogen and the determination of the four diamino acids was carried out on this small quantity. On account of the small amount of material used, less confidence is felt in the result of this particular analysis than in the others. It has been found by experience that the analysis of proteins by van Slyke's method is conveniently carried out on solutions containing about 400 milligrams of nitrogen; in the case of the serum proteins such solutions contain about 100 milligrams of diamino nitrogen. In the case of the proteins of horse serum larger quantities of material were prepared and in three cases enough material was available for duplicate analyses to be carried out.

The results of the analysis of the different proteins of ox and horse serum are summarised in the following tables:

TABLE I.

Summary of the analyses of the different proteins of ox serum, in percentages of the total nitrogen.

	Whole Protein	Albumin	Total Globulin	Pseudo-Globulin	Euglobulin ((NH ₄) ₂ SO ₄ method)	Euglobulin (Panum's method)
Ammonia N ..	7.05	5.8	7.7	7.5	9.3	8.0
Melanin N ..	1.60	1.1	2.0	1.9	2.0	2.5
Cystine N ..	2.75	3.5	2.0	1.9	2.0	1.4
Arginine N ..	11.05	10.4	10.9	10.8	11.6	11.7
Histidine N ..	4.40	6.7	6.3	4.8	3.8	6.5
Lysine N ..	13.55	16.3	9.0	9.6	9.2	9.1
Amino N of the Filtrate	56.65	54.2	59.8	61.7	57.9	58.0
Non-amino N of the Filtrate	2.15	2.3	2.2	1.6	2.8	1.4
Sum ..	99.20	100.3	99.9	99.8	98.6	98.6

TABLE II.

Summary of the analyses of the different proteins of horse serum, in percentages of the total nitrogen.

	Whole Protein	Albumin	Total Globulin	Pseudo-Globulin	Euglobulin ((NH ₄) ₂ SO ₄ method)	Euglobulin (Panum's method)
Ammonia N ..	7.25	6.65	7.95	7.7	7.90	8.6
Melanin N ..	1.60	0.95	2.30	2.2	2.25	2.3
Cystine N ..	2.10	3.10	1.65	1.7	1.65	1.8
Arginine N ..	9.50	9.90	8.00	8.9	8.25	9.4
Histidine N ..	5.60	5.85	4.80	5.8	5.45	5.2
Lysine N ..	12.80	15.90	10.80	9.8	9.95	10.1
Amino N of the Filtrate	59.95	56.55	62.65	61.3	62.20	61.0
Non-amino N of the Filtrate	2.00	1.60	2.15	2.9	2.15	1.8
Sum ..	100.80	100.50	100.30	100.3	99.80	99.6

DISCUSSION OF RESULTS.

From a consideration of the figures given in the two preceding tables it is evident that the composition of serum albumin is very different from that of any of the globulins. Albumin yields on hydrolysis a greater proportion of diamino acids than the globulins, a result which is in agreement with those obtained by Haussmann [1899], Gumbel [1904] and Gibson [1912], who used the nitrogen distribution method devised by Haussmann. Further, albumin

yields a greater proportion of cystine than globulin. In the case of ox serum albumin 3.5 % of the total nitrogen occurs as cystine, while the average for the four globulins is 1.8 %. The corresponding figures for the albumin and globulin from horse serum are 3.1 % and 1.7 %. The results for cystine are in agreement with those of Abderhalden [1903, 1905], who obtained a greater amount of cystine from albumin than from globulin, and of Bywaters and Tasker [1913], who found that the sulphur content of albumin is greater than that of globulin. Estimations of the lysine, arginine, and histidine content of the serum proteins have not hitherto been carried out. From the figures given above for these amino acids it is clear that in the case of both sera examined the albumin differs from the globulins in containing a much larger amount of lysine. It is also to be observed that albumin yields a smaller amount of ammonia and melanin on hydrolysis than the globulins.

Other experimental evidence regarding the lysine content of albumin and globulin has been obtained. Van Slyke and Birchard [1914] determined the free amino nitrogen of a number of proteins and showed that on treatment with nitrous acid in van Slyke's apparatus for half an hour the volume of nitrogen gas evolved corresponded to one half the lysine nitrogen of the protein. Similar experiments have been carried out with the albumin and globulin prepared from ox serum. It was found that albumin yielded about twice as much nitrogen as globulin on treatment with nitrous acid and that in each case the nitrogen obtained was almost exactly one half the lysine nitrogen of the respective proteins. Full details of these experiments will be published shortly.

The marked difference in composition of the proteins of the serum is of interest when considered with reference to the question of the conversion of albumin into globulin, a change which according to Moll [1904, 1906] occurs when serum is heated for an hour at 60° after the addition of a little dilute alkali. Gibson [1912] and Bywaters and Tasker [1913] brought forward evidence which showed that albumin solutions treated in the manner described by Moll yielded a protein which is not identical with naturally occurring globulin and Abderhalden [1903, 1904] showed that globulin yields glycocoll on hydrolysis while albumin does not. The figures given in the above tables show other important differences in the chemical composition of albumin and the globulins of serum, particularly a striking difference in the relative amounts of lysine. When the whole of the evidence dealing with the chemical nature of the serum proteins is considered it is not easy to understand the mechanism of the process by which such simple treatment

of one protein can lead to the formation of another possessing such a widely different chemical composition.

The figures obtained in the analyses of the different globulins indicate that these substances are very similar in chemical composition. Euglobulin was prepared by two methods: (1) by the method of Panum [1851], and (2) from the previously prepared and purified "total globulin"¹ by one-third saturation with ammonium sulphate. A portion of this "total globulin" was set aside for analysis and the remainder was separated into pseudo-globulin and euglobulin by repeated salting out with ammonium sulphate. Analysis of these different globulin preparations by the method of van Slyke failed to reveal any notable differences in chemical composition. The agreement is particularly close in the case of the globulins prepared from horse serum. In the case of the ox serum globulins, the euglobulin prepared by one-third saturation with ammonium sulphate shows a slightly higher ammonia and lower histidine nitrogen value than the other three, but in other respects the agreement is fairly close. As pointed out above, the analytical errors in this particular estimation are probably greater than in the other cases as the analysis was carried out on a much smaller quantity of material. Larger quantities of this protein were prepared from horse serum and the figures obtained on analysis are almost identical with those obtained for the pseudo-globulin and the "total globulin."

The compositions of the different globulins are of interest when considered in connection with the recent work of Chick [1914]. She suggests that the relationship between euglobulin and pseudo-globulin is a very close one, and that under certain conditions pseudo-globulin may undergo a process of "denaturation" whereby a substance is formed the properties of which are very similar to those of euglobulin. She suggests that this artificial euglobulin may be a mechanical complex resulting from the interaction and mutual precipitation of two colloidal systems (viz. pseudo-globulin solution and lipid emulsion) and that the euglobulin of serum may be a protein-lipoid complex of similar origin. The evidence brought forward in this paper regarding the composition of these two proteins lends support to the idea that euglobulin may be related to pseudo-globulin in some such way as Chick suggests. The conversion of pseudo-globulin into euglobulin is a process which, on chemical grounds, would be more easily understood and more capable of explanation than the conversion of albumin into globulin.

¹ The term "total globulin" refers to the protein precipitated from the diluted serum by half saturation with ammonium sulphate. It consists of pseudo-globulin and euglobulin.

EXPERIMENTAL DETAILS.

Preparation of the different serum proteins.

Euglobulin was prepared by the method of Panum [1851], and also by means of ammonium sulphate. Albumin, total globulin, and pseudo-globulin were prepared by salting out with ammonium sulphate. The albumin and globulin were first separated by half saturation with ammonium sulphate. The serum was diluted with four volumes of distilled water and an equal volume of saturated ammonium sulphate solution added. The precipitated globulin was centrifuged off and the solution containing the albumin was saturated with powdered ammonium sulphate. The albumin was filtered off, dissolved in distilled water, and the albumin solution brought to a volume equal to that of the original *diluted* serum. An equal volume of saturated ammonium sulphate solution was added, the precipitated globulin was filtered off, and the solution containing the albumin was again saturated with powdered ammonium sulphate. The albumin was dissolved and reprecipitated five times in all. The albumin solution was always brought to the same volume as that of the original diluted serum before the addition of the saturated ammonium sulphate solution.

The total globulin of the serum was dissolved and reprecipitated five times in the case of ox serum and six times in the case of horse serum. The globulin after each precipitation was dissolved in distilled water and the solution was diluted until the volume was the same as that of the original diluted serum. A portion of the total globulin was then set aside for analysis and the remainder was separated into euglobulin and pseudo-globulin by Haslam's method [1913]. Each globulin was dissolved and reprecipitated five times in the case of ox serum and six times in the case of horse serum globulin.

The different proteins prepared as described above were finally dissolved in distilled water and dialysed against running tap water until free from ammonium sulphate. The aqueous solutions of the different proteins were evaporated to dryness in a modified form of the apparatus described by Martin [1896], a dropping funnel, the exit tube of which was drawn out to a fine capillary, being substituted for the Berkefeldt filter. The bath containing the two drying bottles was maintained at a temperature of 40° and the larger bottle which acts as a receiver was immersed in ice. The dried proteins obtained in this way were reduced to a fine powder and analysed.

Euglobulin was also prepared by the method of Panum [1851]. The serum was diluted ten times with distilled water and normal acetic acid added in sufficient amount to precipitate the euglobulin. The amount of acetic acid necessary for complete precipitation was previously determined by tests on small quantities of the diluted serum. After standing overnight the clear supernatant fluid was decanted and the precipitated euglobulin centrifuged. It was purified by being dissolved in a minimum and measured quantity of decinormal sodium hydrate and reprecipitated by the addition of an exact equivalent of decinormal acetic acid. This process was repeated four times and the euglobulin was finally washed twice in the centrifuge tubes with distilled water.

A sample of the original serum was dried in Martin's apparatus.

Methods of Analysis.

In carrying out the analyses of the different serum proteins the experimental conditions were kept as constant as possible. From four to five grams of material were boiled with 80 cc. of 20 % hydrochloric acid and the rate of hydrolysis was followed by testing samples of the fluid at intervals in van Slyke's apparatus. The hydrolysis was continued for 20 to 22 hours.

After hydrolysis of the protein the excess of hydrochloric acid was removed *in vacuo* and the hydrolysed material washed into a 250 cc. flask. Duplicate quantities of 20 cc. of the solution were used for the determination of the total nitrogen, and 200 cc. of the solution were used in all cases for analysis. The sum of the ammonia nitrogen, melanin nitrogen, total nitrogen of the filtrate, and the total nitrogen of the bases (the last-named being determined in two parts) should be the same as the amount originally present, as determined separately in the 20 cc. portions. The experience gained in the study of these serum proteins shows that with the exercise of ordinary care throughout the course of the analysis it is not difficult to recover the whole of the nitrogen.

In separating the diamino acids from the monamino acids the same amount of phosphotungstic acid (15 g.) was used, and the precipitation was carried out at a volume of 200 cc. in all cases. After the addition of the phosphotungstic acid the mixture was heated on the water bath until most of the precipitate had redissolved, and after cooling to room temperature the vessel was allowed to stand for 48 hours in the cold room. The precipitate of the bases was washed with an ice-cold solution of phosphotungstic acid in hydrochloric acid, 120 to 140 cc. of solution being used.

TABLE III.

Details of observations.

Material	Quan- tity taken g.	Total N		Ammonia		Melanin		Cystine		Argini-
		cc. N/10 acid per 20 cc.	g. N per 200. cc.	cc. N/10 acid per 200 cc.	g. N per 200 cc.	cc. N/10 acid per 200 cc.	g. N per 200 cc.	g. BaSO ₄ per 40 cc.	g. N per 200 cc.	cc. N/10 acid per 100 cc.
A. Ox serum										
1. (a) Whole protein	4	24.2 } 24.1 }	0.3381	16.9	0.0237	3.8	0.0053	0.0240	0.0072	6.2
(b) Whole protein	4	24.5 } 24.6 }	0.3437	17.6	0.0246	4.0	0.0056	0.0209	0.0063	6.1
2. Albumin	4	27.9 } 28.0 }	0.3913	16.2	0.0277	3.3	0.0046	0.0380	0.0114	6.7
3. Total globulin	4	26.5 } 26.4 }	0.3703	20.4	0.0286	5.5	0.0077	0.0170	0.0051	6.7
4. Pseudo-globulin	4	27.2 } 27.2 }	0.3808	20.5	0.0287	5.2	0.0073	0.0160	0.0048	6.8
5. Euglobulin (NH ₄) ₂ SO ₄ method	2.6	16.8 } 16.8 }	0.2352	15.6	0.0218	3.5	0.0049	0.0075	0.0022	4.3
6. Euglobulin Panum's method	4	28.05 } 28.50 }	0.3958	22.7	0.0318	7.0	0.0098	0.0102	0.0031	7.7
B. Horse serum										
7. (a) Whole protein	5.04	33.0 } 32.9 }	0.4613	23.7	0.0332	5.6	0.0078	0.0245	0.0073	7.3
(b) Whole protein	4.95	33.0 } 33.2 }	0.4634	24.3	0.0340	5.0	0.0070	0.0241	0.0072	7.3
8. (a) Albumin	4	29.5 } 29.5 }	0.4130	19.8	0.0277	3.1	0.0043	0.0341	0.0102	6.85
(b) Albumin	4	29.4 } 29.7 }	0.4137	19.5	0.0273	2.9	0.0041	0.0345	0.0103	6.7
9. (a) Total globulin	4	28.4 } 28.2 }	0.3962	22.6	0.0316	6.4	0.0090	0.0170	0.0051	5.2
(b) Total globulin	4	29.2 } 29.4 }	0.4102	23.1	0.0323	6.9	0.0097	0.0115	0.0034	5.2
10. Pseudo-globulin	5	35.7 } 35.6 }	0.4991	27.5	0.0385	7.8	0.0109	0.0203	0.0061	7.4
11. (a) Euglobulin (NH ₄) ₂ SO ₄ method	4.59	32.3 } 32.3 }	0.4522	26.1	0.0365	7.3	0.0102	0.0176	0.0053	6.3
(b) Euglobulin (NH ₄) ₂ SO ₄ method	4.62	32.8 } 32.9 }	0.4599	25.7	0.0360	7.6	0.0106	0.0162	0.0048	6.0
12. Euglobulin Panum's method	4.5	29.5 } 29.5 }	0.4130	23.6	0.0330	6.9	0.0097	0.0159	0.0048	6.4

TABLE III.

Details of observations.

Total N of Bases		Amino N of Bases		Amino N of Filtrate		Total N of Filtrate		Total N recovered
cc. N/10 acid per 100 cc. + arginine titration	g. N per 200 cc.	cc. N per 40 cc.	g. N per 200 cc.	cc. N per 200/15 cc. mm.	g. N per 200 cc.	cc. N/10 acid per 200/6 cc.	g. N per 200 cc.	g. per 200 cc.
35.2	0.0985	22.1; 16°; 768 mm.	0.0648	22.6 } 22.7 } ; 17°; 760	0.1962	24.6 } 24.7 }	0.2071	0.3346
35.0	0.0980	22.5; 18°; 765 mm.	0.0651	22.7 } 22.9 } ; 16°; 768	0.2005	25.45 } 25.50 }	0.2140	0.3422
48.1	0.1347	31.0; 16°; 774 mm.	0.0916	18.2 } 18.4 } ; 15°; 774 ¹	0.2172	20.6 } 20.7 }	0.2313	0.3933
34.1	0.0955	18.7; 17°; 763 mm.	0.0542	26.3; 19°; 762	0.2265	28.6 } 28.4 }	0.2394	0.3712
33.5	0.0938	19.2; 18°; 764 mm.	0.0555	28.0 } 27.8 } ; 19°; 762	0.2400	29.9 } 29.9 }	0.2512	0.3810
18.8	0.0526	10.6; 15°; 764 mm.	0.0310	16.1 } 16.0 } ; 15°; 764	0.1415	18.3 } 18.1 }	0.1529	0.2322
37.1	0.1039	19.5; 16°; 764 mm.	0.0568	20.0 } 20.3 } ; 16°; 764 ¹	0.2349	22.0 } 21.8 }	0.2453	0.3908
46.5	0.1302	29.5; 22°; 770 mm.	0.0843	32.4 } 32.6 } ; 20°; 773	0.2820	35.2 } 35.2 }	0.2957	0.4669
45.8	0.1282	29.2; 21°; 765 mm.	0.0832	32.9 } 33.0 } ; 21°; 768	0.2829	35.5 } 35.4 }	0.2978	0.4670
48.25	0.1351	31.6; 17°; 766.5 mm.	0.0184	27.3 } 27.3 } ; 17°; 766	0.2385	30.0 } 29.9 }	0.2516	0.4188
47.2	0.1321	31.3; 17°; 766.5 mm.	0.0182	27.5 } 27.6 } ; 18°; 766	0.2394	29.7 } 29.7 }	0.2495	0.4130
33.2	0.0930	20.8; 20°; 770 mm.	0.0600	28.8 } 28.9 } ; 19°; 773	0.2517	31.7 } 31.7 }	0.2663	0.3999
32.7	0.0916	20.9; 20°; 764 mm.	0.0598	30.5 } 30.4 } ; 19°; 770	0.2640	32.9 } 33.0 }	0.2768	0.4104
43.3	0.1212	25.8; 21°; 764 mm.	0.0734	36.7 } 36.7 } ; 22°; 763	0.3114	39.4 } 39.4 }	0.3309	0.5015
37.1	0.1039	22.8; 18°; 752 mm.	0.0648	33.6 } 33.4 } ; 18°; 752	0.2856	35.8 } 36.0 }	0.3015	0.4521
38.4	0.1075	23.4; 20°; 762 mm.	0.0667	34.2 } 34.4 } ; 18°; 752	0.2925	36.4 } 36.5 }	0.3062	0.4603
35.5	0.0994	21.9; 24°; 757 mm.	0.0608	31.1 } 31.1 } ; 25°; 755	0.2572	32.0 } 32.2 }	0.2696	0.4117

¹ Solution brought to 200 cc.

One of the more difficult of the estimations in the process is the determination of the total nitrogen of the bases, which is carried out on the same solution used for the determination of the arginine. In these analyses the practice was adopted of continuing the digestion for some time after the solution becomes clear, gently moving the fluid round the sides of the flask from time to time in order to wash down into the acid any particles which may have crept on to the upper parts of the digestion flask. The figures for the histidine nitrogen show the widest variation in duplicate analyses. This value is not determined directly but is calculated from the results of three other determinations, viz. the arginine nitrogen, the amino nitrogen of the bases, and the total nitrogen of the bases. As van Slyke points out these three values can all be determined accurately, and the duplicate analyses for the histidine should not vary by more than one per cent. of the total nitrogen.

The following estimations were carried out on the solution of hydrolysed protein :

- (1) Total nitrogen was estimated in 20 cc. of the original solution.
- (2) Ammonia nitrogen and melanin nitrogen were estimated in 200 cc. of the original solution.
- (3) After removal of the ammonia and melanin the separation with phosphotungstic acid was carried out.
- (4) The filtrate from the phosphotungstic acid precipitate of the bases was made to 150 cc. and the following estimations made :
 - (a) Total nitrogen of the filtrate in 25 cc.
 - (b) Amino nitrogen of the filtrate in 10 cc.
- (5) The solution of the bases was made to 50 cc. and the following estimations made :
 - (a) Cystine nitrogen in 10 cc.
 - (b) Amino nitrogen in 10 cc.
 - (c) Arginine nitrogen and total nitrogen of the bases in the same sample of 25 cc.

The detailed figures obtained in the analysis of the different serum proteins are given in the tables. Table III (pp. 548, 549) contains a record of the actual observations, the volumes given in the headings referring in every case to the amount of the original solution corresponding to the volume actually employed as explained above. In Table IV the results are expressed as percentages of the total nitrogen.

TABLE IV.

Results of analyses expressed in percentages of total nitrogen.

Material	Ammonia N	Melanin N	Cystine N	Arginine N	Histidine N	Lysine N	Amino N of Filtrate	Non-Amino N of Filtrate	Total N recovered
A. Ox serum									
1. (a) Whole protein	7.0	1.6	2.1	10.2	3.4	13.3	58.0	3.2	98.8
Corrected for solubility of bases			2.9	11.2	4.5	13.4	56.5	1.8	
(b) Whole protein	7.1	1.6	1.8	9.9	3.2	13.5	58.3	3.9	99.3
Corrected			2.6	10.9	4.3	13.7	56.8	2.5	
2. Albumin	5.8	1.1	2.9	9.6	5.7	16.2	55.5	3.6	100.4
Corrected			3.5	10.4	6.7	16.3	54.2	2.3	
3. Total globulin	7.7	2.0	1.4	10.1	5.3	8.9	61.1	3.4	99.9
Corrected			2.0	10.9	6.3	9.0	59.8	2.2	
4. Pseudo-globulin	7.5	1.9	1.2	10.0	3.9	9.5	63.0	3.0	100.0
Corrected			1.9	10.8	4.8	9.6	61.7	1.6	
5. Euglobulin (NH ₄) ₂ SO ₄ method	9.3	2.0	0.9	10.2	2.2	9.0	60.1	4.9	98.6
Corrected			2.0	11.6	3.8	9.2	57.9	2.8	
6. Euglobulin Panum's method	8.0	2.5	0.8	10.9	5.6	9.0	59.3	2.6	98.7
Corrected			1.4	11.7	6.5	9.1	58.0	1.4	
B. Horse serum									
7. (a) Whole protein	7.2	1.7	1.6	8.8	4.9	12.8	61.1	3.0	101.1
Corrected			2.1	9.5	5.8	12.9	60.0	1.9	
(b) Whole protein	7.3	1.5	1.5	8.8	4.6	12.6	61.0	3.2	100.5
Corrected			2.1	9.5	5.4	12.7	59.9	2.1	
8. (a) Albumin	6.7	1.0	2.4	9.3	5.1	15.7	57.7	3.1	101.0
Corrected			3.1	10.0	6.1	16.0	56.5	2.0	
(b) Albumin	6.6	0.9	2.5	9.0	4.6	15.7	57.8	2.4	99.5
Corrected			3.1	9.8	5.6	15.8	56.6	1.2	
9. (a) Total globulin	8.0	2.3	1.3	7.3	4.1	10.6	63.5	3.7	100.8
Corrected			1.9	8.1	5.1	10.7	62.2	2.4	
(b) Total globulin	7.9	2.3	0.8	7.1	3.6	10.7	64.3	3.1	99.8
Corrected			1.4	7.9	4.5	10.9	63.1	1.9	
10. Pseudo-globulin	7.7	2.2	1.2	8.3	5.0	9.7	62.4	3.9	100.4
Corrected			1.7	8.9	5.8	9.8	61.3	2.9	
11. (a) Euglobulin (NH ₄) ₂ SO ₄ method	8.0	2.2	1.1	7.8	4.2	9.8	63.1	3.5	99.7
Corrected			1.7	8.5	5.0	9.9	62.0	2.4	
(b) Euglobulin (NH ₄) ₂ SO ₄ method	7.8	2.3	1.1	7.3	5.1	9.9	63.6	3.0	100.1
Corrected			1.6	8.0	5.9	10.0	62.4	1.9	
12. Euglobulin Panum's method	8.0	2.3	1.1	8.7	4.3	10.0	62.3	3.0	99.7
Corrected			1.8	9.4	5.2	10.1	61.0	1.8	

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XXIV. THE FREE AMINO NITROGEN OF THE DIFFERENT PROTEINS OF OX AND HORSE SERUM.

BY PERCIVAL HARTLEY.

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(Received May 11th, 1915.)

In a recent communication van Slyke and Birchard [1913] have shown that when certain native proteins are treated with nitrous acid the amount of nitrogen formed as a result of the reaction is equal to one-half the lysine nitrogen of the protein. They suggest that one of the amino groups of lysine, the ω -group, exists free in the intact protein molecule, and, moreover, that this ω -amino nitrogen of the lysine represents practically the entire amount of free amino nitrogen which can be demonstrated by the nitrous acid method of van Slyke. All the amino acids, except lysine, react quantitatively with nitrous acid in three to four minutes; lysine reacts more slowly, thirty minutes being required for complete reaction, and van Slyke and Birchard show that reaction between nitrous acid and the native proteins also requires about thirty minutes to attain completion. The authors quote observations of other workers which support their view that the free amino nitrogen of native proteins is due to the ω -amino group of the lysine which they contain. Gelatin, caseinogen and serum globulin after treatment with nitrous acid yield no lysine on subsequent hydrolysis with acid. Clupeine, which contains no lysine, yields no nitrogen on treatment with nitrous acid, while sturine and cyprinine, which contain lysine, do. It has also been shown that certain protamines and proteins which contain lysine reveal free amino groups by the formal titration method of Sørensen, while those which contain no lysine do not.

The analysis of the different proteins of ox and horse serum, an account of which has recently appeared [Hartley, 1914], shows that albumin differs in several important respects from the globulins. In particular it has been found that albumin contains a much larger amount of lysine than the globulins. The experiments described in this paper have been carried out in order to study the relationship between the free amino nitrogen of the different serum proteins and their lysine content.

The preparation of the different serum proteins has been described in the earlier paper. For the determination of the amino nitrogen the following procedure was adopted in each case. Two weighed quantities of the air-dried preparations were taken: one of these was used for the determination of the total nitrogen of the protein by Kjeldahl's method and the other was dissolved in water or very dilute alkali in a standard 15 cc. flask. Ten cc. of this solution were treated with nitrous acid in van Slyke's apparatus and the free amino nitrogen of the protein determined. The reaction was conducted in the standard size apparatus described by van Slyke [1912], 1 cc. of octyl alcohol being added in each case to prevent frothing. The mixture of nitrous acid and protein was shaken at intervals for twenty-five minutes and finally shaken continuously for five minutes.

The results of the determination of the total nitrogen and the free amino nitrogen are collected in the following tables.

Total globulin is the name given to the proteins precipitated from the diluted serum by half saturation with ammonium sulphate. It consists of pseudoglobulin and euglobulin. Euglobulin *A* was prepared from the total globulin by salting out with ammonium sulphate, and euglobulin *P* was prepared by the dilution and acidification method of Panum.

TABLE I.

Total Nitrogen Determinations.

No.	Protein	Quantity taken g.	N/10 H ₂ SO ₄ neutralised cc.	Nitrogen mgm.
1.	Albumin (ox)	0.2065	18.8	26.32
2.	Total globulin (ox)	0.2110	19.9	27.86
3.	Albumin (horse)	0.2562	23.7	33.18
4.	Total globulin (horse)	0.2604	24.9	34.86
5.	Pseudoglobulin (horse)	0.2882	27.1	37.94
6.	Euglobulin <i>A</i> (horse)	0.2894	26.4	36.96
7.	Euglobulin <i>P</i> (horse)	0.3047	25.2	35.28
8.	Whole proteins (horse)	0.2761	23.3	32.62

TABLE II.

Amino Nitrogen Determinations.

No.	Protein	Quantity taken gm.	N gas cc.	Temp. °C.	Pressure mm.	Amino N mgm.	Per cent. of total N as free NH ₂
1.	Albumin (ox)	0.5157	9.9	16	755	5.70	8.7
2.	Total globulin (ox) ..	0.5013	4.8	16	758	2.80	4.2
3.	Albumin (horse)	0.3875	7.0	17	762	4.05	8.0
4.	Total globulin (horse)	0.3932	5.2	19	762	2.98	5.6
5.	Pseudoglobulin (horse)	0.2806	3.1	15	760	1.81	4.6
6.	Euglobulin A (horse)	0.4096	5.4	17	749	3.07	5.8
7.	Euglobulin P (horse)	0.4050	4.3	17	749	2.44	5.2
8.	Whole protein (horse)	0.3963	5.1	19	762	2.92	6.2

In Table III the results of the determination of the free amino nitrogen are given and compared with the lysine content of the different proteins. The figures for the lysine are taken from the earlier paper [1914] dealing with the analysis of the different proteins of ox and horse serum.

TABLE III.

No.	Protein	Per cent. of total nitrogen as	
		Free amino N	Half lysine N
1.	Albumin (ox)	8.7	8.15
2.	Total globulin (ox)	4.2	4.50
3.	Albumin (horse)	8.0	7.95
4.	Total globulin (horse) . . .	5.6	5.40
5.	Pseudoglobulin (horse) . . .	4.6	4.90
6.	Euglobulin A (horse)	5.8	4.97
7.	Euglobulin P (horse)	5.2	5.05
8.	Whole proteins (horse) ..	6.2	6.40

Except in the case of Euglobulin A (horse) in which the result is a little high, the free amino nitrogen of the proteins of ox and horse serum is equal to one-half the lysine nitrogen. The results are in agreement with those obtained by van Slyke and Birchard for other native proteins and lend support to the suggestion advanced by them that in the intact protein molecule the ω -amino group of the lysine exists free, and, further, that this group represents practically the whole of the free amino nitrogen determinable by means of nitrous acid.

The results given above, taken in conjunction with those of van Slyke and Birchard, indicate that the lysine content of a native protein may be rapidly estimated by determining the amino nitrogen and the total nitrogen of the protein.

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**SOME DETAILS IN THE ANATOMY OF THE RAT-FLEA,
CERATOPHYLLUS FASCIATUS BOÏSC.**

BY PROF. E. A. MINCHIN, M.A., HON. PH.D., F.R.S.

(Read January 26th, 1915.)

PLATES 26-32.

DURING the past five years I have been engaged, in collaboration with a friend, upon investigations, recently published,* into the development of the rat-trypanosome in its invertebrate host the rat-flea (*Ceratophyllus fasciatus*). In the course of this investigation we have dissected and examined some 1,700 fleas; and although these dissections were not undertaken with the primary object of studying the anatomy of the flea, but only with the intention of extracting and examining those organs of the flea likely to contain stages of the trypanosome, it goes without saying that we have not pulled so many fleas to pieces without gaining some insight into the structure of the insect, and it seemed to me worth while to study some anatomical points of structure in more detail and in special preparations. Some parts of the flea are very interesting as regards their structural relations and make very beautiful microscopic preparations which can be mounted with very little trouble. I thought it might interest the members of the Club if I laid before them a brief account of some points of flea-anatomy seen by the way—*obiter visa*, if I may use the expression.

Before describing these observations, I wish it to be clearly understood that this paper does not pretend to give a complete anatomical description either of the flea as a whole or even of the systems of organs that are dealt with. There are many structural details which could only be made out by sections, and I have had no leisure for the task of section-cutting, always a difficult and laborious undertaking in the case of insects, on account of the toughness of the chitinous cuticle, which cannot

* Minchin and Thomson, "The Rat-trypanosome, *Trypanosoma lewisi*, in its Relation to the Rat-flea, *Ceratophyllus fasciatus*." *Quarterly Journal of Microscopical Science*, Vol. LX., Part 4, 1915.

be dissolved out. I do not propose to describe any details here which cannot be verified by an observer possessing a dissecting microscope* and a pair of mounted dissecting needles—and a flea! In fact the results obtained by me and set forth here are based entirely on what may be termed “needlework.”

Before proceeding to anatomical descriptions, I may give a brief account of the technique I have employed. The flea at liberty is, I need not say, an active and elusive insect. But when placed on the surface of water, he is perfectly helpless, and floats there without being able to escape and without drowning for at least twenty-four hours, provided there is no soap in the water; if there is a trace of soap the cuticle of the flea is wetted and the insect sinks and is soon drowned. (This hint may be borne in mind as being often useful in the home.)

Having therefore caught your flea, put it on the surface of some water and keep it until you can proceed further with your operations. An expeditious way of catching the flea is to get it to hop straight on to the surface of water. In doing this remember that a flea always hops by preference away from the source of light, never towards it.

When it is desired to dissect the flea it should be gathered off the surface of the water with a fine forceps and placed in a drop of physiological salt-solution (0.75 gramme sodium chloride in 100 cubic centimeters of distilled water) on an ordinary microscopical slide, which is then placed on the stage of the dissecting microscope.

For the dissection I use two fine needles mounted in wooden handles. Each needle after fixing in the handle is ground down further on an ordinary hone. One of them is ground to a fine sharp point, the other to a flat cutting edge. For preparing the flat-edged needle, I first take a penknife and pare the extremity of the wooden handle on both sides so that it is shaped like an ordinary brad-awl. I then rub the needle down on the hone in two planes parallel to the two cuts made in the handle, checking the process under the dissecting microscope and trying to get a rounded cutting edge, not an edge which terminates in a straight line like an ordinary chisel. The object of paring the wooden handle is both to guide the hand when rubbing down the needle

* I have used in all my work a Greenough binocular dissecting microscope made by Zeiss.

on the hone, and also to distinguish the flat-edged needle from the pointed one. When dissecting I use the pointed needle in my left hand for holding the object, and the flat-edged needle in my right hand for cutting. The needles should be pushed far into the wooden handle, so that only a short length is free, otherwise the needle is too springy and is liable to snap under pressure.

The flea was left in the drop of salt-solution, where it is kicking about violently and may succeed, if not watched, in getting out on to the slide and hopping off. It is therefore best to begin by decapitating the flea. This can be done by holding it still with the pointed needle and snipping off the head with the flat-edged needle. The dissection can then be proceeded with in a manner free from haste or anxiety.

I will describe now a method of making permanent preparations of the organs of the flea which I have found very useful. There is not a single detail of anatomy described in this paper which I could not demonstrate to a sceptic in my permanent preparations* at a moment's notice. Let us take the abdominal nervous system, for example. The complete nervous system of the flea consists, as in other insects, of the three sets of nerve-ganglia: (1) the cephalic ganglion-complex, or brain, situated in the head dorsal to the digestive tract (supra-oesophageal); (2) three pairs of thoracic ganglia, corresponding to the three thoracic segments: (3) a chain of abdominal ganglia extending into the abdomen. Parts (2) and (3) are ventral to the digestive tract and constitute a continuous chain of pairs of ganglia, but the two ganglia of any given pair are fused together so as to appear like a single ganglion-mass. Each pair of ganglia is connected with the pair next behind or in front by a pair of stout nerves, known as "connectives," and it can be plainly seen that these connectives remain distinct in each pair, and are not fused together like the ganglion-pairs (Pl. 26). The first pair of thoracic ganglia is connected with the brain by a pair of peri-oesophageal connectives. From the ganglia are given off nerves to the various organs of the body.

It is almost impossible to dissect out the brain, and to study its structure and relations sections would be necessary. It is difficult, but by no means impossible, to dissect out the thoracic ganglia. Major Christophers, I.M.S., who worked for a time in my

* These preparations are now the property of the Club.

laboratory at the Lister Institute, made some beautiful dissections of the ventral nervous system of the flea, showing both thoracic and abdominal ganglia in continuity. On the other hand, it is by no means difficult to dissect out the abdominal chain in its whole length, up to and including the large metathoracic ganglia, the ganglia of the jumping legs. To do this the flea should be held by the thorax with the pointed needle, while with the flat-edged needle the abdominal segments are carefully detached and pulled off from behind forwards successively, until only the thoracic segments are left. If the operation has been successfully performed, and the abdominal segments together with the contained digestive and reproductive organs removed, the abdominal chain of ganglia will be seen proceeding from, and adhering to, the hindmost thoracic segment. With practice the complete severance of the abdomen and its organs from the thorax can be effected with one pull.

Now take another slide and place on it a cover-slip ($\frac{3}{4}$ inch square). Place the slide and cover-slip on the stage of the dissecting microscope, put a quite small drop of salt-solution on the cover-slip, and transfer the thorax of the flea from the slide on which it was dissected to the small drop of fluid on the cover-slip, and there proceed with the dissection. The big metathoracic ganglion-mass can be seen quite plainly in the hindermost part of the thorax, with the abdominal chain of ganglia proceeding from it. With the needles the metathoracic ganglion must be carefully dissected out and set free from the thorax; this operation is not at all difficult, though it requires both skill and practice to dissect out the first two thoracic ganglia as well, in unbroken continuity with the rest of the ganglionic chain. If during the dissection the cover-glass slips about on the slide, it can be fixed quite firmly by letting a tiny drop of distilled water run in between cover-glass and slide, but I avoid this as a rule, because it makes it difficult to get the cover-slip off later on.

When the dissection has been completed, the fragments and debris of the thorax should be removed and cleaned up as much as possible, leaving the nervous system in the small drop of fluid on the cover-slip. Now the cover-slip must be lifted carefully off the slide and all superfluous moisture drained off it, so as to leave the nervous system stranded on the cover-slip, as near the centre as possible. The fluid can be drained off either by tilting the

cover-glass and letting the salt-solution run off, or, if the nervous system shows a tendency to run off with the fluid, by holding the cover-glass flat and carefully mopping up all superfluity of fluid with a small piece of filter-paper. The object to be attained is to leave the specimen stranded on the cover-glass and to drain off as much of the salt-solution as possible, in order that by capillary attraction the object may be pressed against the cover-slip; but on no account must the fluid be allowed to dry completely. When this has been done the cover-slip is inverted, so that the object is on its lower side; and then it is dropped face downwards quite flat on to the surface of some fixative fluid.

Various fixatives can be used, but I have nearly always made use of 5 per cent. sublimate-acetic—that is to say, saturated solution of corrosive sublimate in distilled water, 95 volumes, mixed with glacial acetic acid, 5 volumes. When fixing the preparation some of the fixative is put into a large watch-glass or clock-glass and the cover-glass with the adherent object is dropped on to it and remains floating on the surface of the solution. In nine cases out of ten the object remains firmly adherent to the under side of the cover-glass, if one has hit the happy medium in draining off the fluid in which it was dissected out. If superfluity of the salt-solution remains, the object will come off; if it has been allowed to dry up altogether, the preparation is ruined.

The cover-glass with the adherent organs can now be manipulated just as if it was a smear, lifting the cover-slip with an ordinary forceps and transferring it from one liquid to another. After the preparation has been fixed in the sublimate-acetic for some time, say from 10 minutes to an hour, it can be brought up through successive strengths of alcohol in watch-glasses (10 per cent., 30 per cent., 50 per cent. and 70 per cent.) to 90 per cent. alcohol, in which it should be left for a longer time (preferably over night, or as long as is convenient) in order that the preparation may be well hardened and the corrosive sublimate thoroughly dissolved out. In the stronger alcohols the cover-slip will sink, but it rests on its corners on the rounded bottom of the watch-glass and there is no contact or pressure on the object, which of course is on the under side of the cover-slip. It is now apparent why square cover-slips must be used, since they rest on their corners and can be easily picked up with the forceps;

round cover-slips would be in contact with the watch-glass round their whole edge, and be very troublesome to lift up with the forceps or in any other way.

Sometimes the object comes away loose from the cover-slip in the sublimate-acetic. When this annoying event takes place, put the cover-slip into a watch-glass and cover it with 30 per cent. alcohol; then draw up the object from the sublimate-acetic mixture with a glass pipette of sufficiently wide calibre and place it on the upper surface of the cover-slip in the alcohol. Then lift up the cover-slip carefully with a forceps, taking care the object does not float off the cover-slip to one side or the other, but remains stranded on the cover-slip again. Then drain off the alcohol, invert the cover-slip, and drop it face downwards into 50 per cent. alcohol in another watch-glass. This time the rebellious object always sticks to the cover-slip. In all cases the cover-slips should be handled delicately while in the sublimate-acetic or in the weak alcohols, since a too violent jerk may dislodge them; but I have never known an object to come loose after it has got so far as the 70 per cent. alcohol.

I have described this method in full detail because I have found it extremely useful for making permanent preparations of dissections. In the flea, for example, it is very easy to dissect out and mount in this way the entire male reproductive system, from testes to penis, and so display every detail of it; and since the preparation is adherent to the cover-slip, any powers of the microscope, even immersion lenses, can be focused on to it for study of minute details. The principle of the method is that cellular tissues, having been pressed firmly but gently against the glass by capillary attraction, adhere to the glass by their own stickiness; and when the preparation has been well fixed and hardened, the coagulation of the albumins glues the organs so firmly that they cannot be detached without breaking them. Naturally this does not apply to chitinous organs, which are not wetted by water, and can never be made to stick in this way.

The preparations, after having been fixed and hardened, can be mounted unstained, or can be stained first in any way desired. Unstained preparations are best for showing internal details of the chitinous cuticle or skeleton; stained preparations for showing the cellular structure of the tissues and soft parts; the

one method of preparation supplements the other. For staining an alcoholic stain is preferable, since prolonged soaking in watery stains might produce maceration and cause the object to become detached again from the cover-slip. I have always used Grenacher's alcoholic borax-carminé, in which the objects are stained for about five minutes, and then transferred to acidulated alcohol (0.1 per cent. hydrochloric acid in 70 per cent. alcohol), in order to extract all the carminé stain from the cytoplasm of the cells and leave it only in the nuclei. If the stain be not thoroughly extracted in this way the preparation will be very opaque, and I find it best to leave the objects in the acidulated alcohol for about forty-eight hours, changing the fluid occasionally. I believe this method could be improved upon, and that Mayer's alcoholic paracarminé* would give a more transparent stain, and one more easily extracted. Some of the well-known hæmatoxylin mixtures would probably also give good results.

The stained or unstained preparations are then finished off by passing them into absolute alcohol, then into oil of cloves or any other of the ordinary clearing reagents, and finally into Canada balsam. The cover-slips can be mounted over well-slides or—preferably, in my opinion—on ordinary slides, with the precaution of supporting the corners of the cover-slip on wax feet or in some other way, in order that the objects may run no risk of being crushed between slide and cover-slip.

I will now proceed to set forth some of my observations on the anatomy of the flea, noting, as a preliminary, that all my statements apply to the common rat-flea, *Ceratophyllus fasciatus*, the only species I have dissected. Other species of flea may perhaps show slight differences in some points.

It is also my pleasant duty, at this point, to express my warm thanks to Miss Mabel Rhodes, artist at the Lister Institute, for kindly executing the drawings of my dissections which accompany this paper. They were all drawn with the camera lucida from the actual preparations.

I. *The Abdominal Nervous System.*

The method of dissecting out the abdominal chain of nerve-ganglia has been described above. It is one of the easiest

* For an account of these stains and how to prepare them see Bolles Lee's well-known *Vade-mecum*.

dissections if one is content to get out only the large metathoracic ganglion-mass, in the thoracic series, and not to worry about the ganglia of the first two thoracic segments, which require very careful dissection.

A remarkable feature of the abdominal nervous system is that it presents very marked differences in the two sexes of the flea. These sexual differences are seen at a glance in the two figures on Pl. 26, which are drawn from two preparations to the same scale by means of a camera lucida. At the upper end of each figure we see the large metathoracic ganglion-mass, and at the lower end the large hindmost or terminal ganglion-complex from which nerves are given off to the genitalia. Between these two larger nerve-centres at the two extremities there is a series of smaller ganglia; and it is easy to see that this series comprises seven ganglia in the male and only six in the female.*

It is seen, then, that the male flea has one pair of ganglia more in its abdominal nervous system than the female. Is this an indication of superiority on the part of the male sex? By no means, rather the contrary! In the embryonic development of insects there are some ten or eleven pairs of abdominal ganglia, and in the ontogenetic development, or in the phylogenetic evolution, of insects the tendency is for these ganglia to be concentrated by fusion which takes place progressively from behind forwards. In some of the Diptera—the tsetse-fly, for example, and I believe in the common house-fly also—the concentration of the nerve-ganglia has reached its maximum possible, since the whole ventral chain is concentrated into one large mass situated in the thorax, a mass which represents the three pairs of thoracic ganglia plus the whole abdominal chain, all telescoped forwards into one large ganglion-complex. In the flea, however, the process of concentration and specialisation has not gone so far, and is seen only at the hindmost end of the

* This curious point was discovered by Major Christophers, in his dissections of fleas made in my laboratory. Previous to his work, I had counted the ganglia of a female flea that I was dissecting, and had noted that there were six small ganglia. Subsequently I made a mounted preparation of the abdominal chain of a male flea, and was surprised to observe seven small ganglia; thinking I had made a mistake in my former observation, I looked up my old notes and altered "six" to "seven," never suspecting the sexual differences which were subsequently shown to exist.

nervous system, in the large terminal ganglion-mass, which represents a fusion of the most posterior ganglia. The difference in the number of the abdominal ganglia in the two sexes of the flea shows, therefore, that in the female the concentration has gone one step farther than in the male, since only six abdominal ganglia remain free in the female, but seven in the male. The nervous system of the female has therefore reached one stage in evolution higher in the female than in the male. Similar differences between the sexes are known to occur also in other insects, especially in the Hymenoptera (the order which includes the bees, ants, and wasps), an order in which the superiority in intelligence and in the social virtues of the female over the male is very marked.

Besides the difference in the number of ganglia, the nervous systems of the male and female flea differ also in the arrangement of the nerve-stems given off from the hindmost ganglion-mass. In the male two stout nerves are given off, which run on either side of the "corkscrew-organ" (see p. 454), and are distributed mainly to the powerful muscles which work the penis. In the female, however, three pairs of moderately stout nerves are given off, which go to the genitalia, but I have not been able to trace their exact distribution.

Comparing the two figures, it is seen that the male and female nervous systems are approximately of the same absolute length. Since, however, the female flea is considerably larger than the male, the nervous system of the female is relatively much the shorter, and does not extend so far into the abdomen as that of the male. Consequently the nervous system of the male is the easier to dissect out.

As regards minuter details, the nerve-ganglia are seen to contain a number of nuclei, representing the ganglion-cells, which have a bilaterally symmetrical arrangement, showing that each ganglion-mass is a fusion of a pair of ganglia. The nerves which come off from the ganglia right and left contain small, elongated nuclei, which are the nuclei of the connective tissue-sheaths of the nerves. The connectives running between the successive abdominal ganglia contain no nuclei, but the stout connectives passing forwards from the metathoracic ganglion-mass contain elongated nuclei similar to those of the peripheral nerves.

II. *The Salivary Glands.*

Having occasion to dissect some flea-larvae, I was struck by the fact that the salivary glands of the larva differ greatly, both in size and in complication of parts, from those of the adult flea. I will begin with the adult, in which the glands are both smaller and simpler in structure.

In the adult flea the salivary glands lie in the abdomen, right and left of the stomach, in the form of two tiny pouches on each side (Pl. 27, B and C). Each pouch consists of large glandular cells, which tend to stain very opaquely and have large nuclei. The two pouches of each side give off each a short duct, and these two ducts unite into a long duct running forwards on the side of the body to the anterior thoracic region, where the two ducts from the two sides of the body unite into a common salivary duct, which runs forwards to open, doubtless, into the hypopharynx, as in other insects. The paired salivary ducts have a very characteristic appearance, being lined by a chitinous cuticle which shows internally a system of rather irregular transverse thickenings. This appearance is seen from the point where the ducts issue from the glands up to a short distance from the spot where the paired ducts unite to form the common salivary duct; the structure of the ducts recalls to some extent that of a tracheal tube, but the transverse thickenings are not so perfectly regular as in the tracheae. At the point of union of the right and left salivary ducts, however, there is a Y-piece in which the duct diminishes in calibre to about half, and has no transverse thickenings. External to the chitinous lining, the duct is covered by a delicate layer of flat epithelium, which does not show distinct cell-outlines, but has the appearance of a plasmodial or syncytial layer of protoplasm with scattered nuclei.

The salivary gland of the adult flea, on account of its small size, is not so easy to dissect out; the glands of the female are slightly larger than those of the male. On the other hand, the salivary glands of the larva, which are plainly visible through the body-wall of the living insect, are very easily dissected out. All that is necessary is to decapitate the larva in such a way as to cut off the first or first two thoracic segments, together with the head, and then to press with the flat of a dissecting needle gently along the body from behind forwards, so as to squeeze out

the contents of the body-cavity through the cut end of the trunk. The salivary glands sometimes come out as soon as the flea is decapitated, without any such pressure, and it is easy to get them on to a cover-slip and fix them.

Almost the only point in which the larval glands (Pl. 27, A) resemble those of the adult is in the characteristic structure of the duct, which can be recognised immediately. Passing back along the duct (d.), we come to a thin-walled dilated sac or reservoir (r.), quite absent in the adult. Behind the duct a tubule begins, composed of lightly staining glandular cells. After a short course this tubule becomes continuous with the gland proper, which is composed of darkly staining glandular cells, and branches out into three lobes or diverticula, two of which run forward (l.a.¹, l.a.²) and one backward (l.p.) alongside of the digestive tract. All this arrangement of duct, reservoir, and gland is, of course, duplicated on each side of the body, right and left.

Accompanying the larval salivary gland are two elongated pads or cushions of fat-body, which are very difficult to separate from the gland without damaging the glandular lobes. In the hinder of these pads of fat I found in many fleas a body which looked exceedingly like a parasitic cyst, for which I mistook it at first. Specimens mounted whole showed the "cyst" to be composed of large cells in the interior, showing a tendency in the more advanced specimens to arrangement in longitudinal rows, and enveloped by a layer of flat epithelium at the surface. At its hinder end the "cyst" is prolonged into a delicate cord of cells which could be traced in some specimens a long way back. Further investigation showed, however, that when this "cyst" was present on one side of the body it was also present on the other side in exactly the same degree of development; and further, that when the "cysts" were absent in the fat-body on the level of the salivary glands, they were to be found in other pads of fat-body situated farther back, on the level of the intestine right and left. Hence it was obvious that the supposed parasitic cysts were simply the genital rudiments, situated farther forward in the larvae of one sex than in the other. Whether it is the male, or the female, in which they are situated farther forward, I cannot say.

The striking differences between the larval and adult flea in

respect to the salivary glands must be related to the difference in their habits. The adult flea, I need not say, is a blood-sucker, and in blood-sucking insects generally the function of the salivary glands is believed to be that of producing a secretion which is mixed with the ingested blood and prevents it from coagulating. Incidentally the salivary glands of the adult flea, if crushed and examined, can be seen to contain many yeast-like bodies of several kinds, and it is supposed that it is these microbes which are responsible for the local irritation and itching caused by the puncture of the flea's proboscis. The flea-larva, on the other hand, is more or less omnivorous, but appears to feed principally on the faeces of the rat, as well as dirt and debris of all kinds. Consequently its salivary glands have a function in the insect's economy entirely different from that of the adult flea, assisting probably in the digestion of the food, and their larger size in the larva indicates a greater secretive activity than in the adult.

III. *The Male Reproductive Organs.*

The genitalia of the male flea exhibit a singular complication of parts and of their arrangement, but are nevertheless very easy to dissect out, and with a little care the entire reproductive system, from testes to penis, can be mounted as one preparation, in which every detail can be studied with the exception of those minuter points of structure which require sections for exact study.

A general sketch of the various parts is given in Plate 28. All the details of this sketch have been drawn from mounted dissections with the camera lucida at a magnification of 150 diameters, reduced in the reproduction by one-half. At the same time the relation of the various parts and their relative position in the body has been checked by sketches of the whole system, both of such parts of it as can be seen through the body-wall of the flea without dissection, and also as it is seen when the abdomen of the flea is freshly opened with the least possible disturbance of the organs.

Most anteriorly are situated the two conspicuous testes (T., T.) with their ducts coming off from them, and shaped somewhat like a pear would be if the stalk (the duct) came off from its thicker end. The testes lie dorsal to the stomach, but vary to some extent both in size and arrangement. When the testes are of large size, as in the younger males, they lie one in front of the

other, and then the duct of the testis lying more anteriorly runs straight back, while that of the testis situated more posteriorly is coiled. When the testes are smaller, as in the older, more exhausted males, they lie side by side and their ducts run straight back.

When the testis is examined it is seen at once to consist of two parts, a dilated bladder-like portion of ovoid shape, at the base of which is a coiled tubular portion. The bladder-like portion appears to be the testis proper (T.), while the coiled tubular portion (ep.¹) recalls the structure in the human testis known as the epididymis, and may be known conveniently by this designation. In one of my dissections I succeeded in uncoiling the epididymis forcibly, by pulling on the duct (ep.²). It was then seen that the epididymis is a thin-walled tube, filled with ripe spermatozoa; consequently, from the point of view of function, the epididymis represents a vesicula seminalis, that is to say a receptacle for the storage of ripe sperm.*

The calibre of the tubular epididymis narrows rapidly as it passes on into the duct, which may be called here, as in other animals, the vas deferens. The right and left vasa deferentia (v.d.¹, v.d.²) run back a little way and join to form the common vas deferens (v.d.³), but it can be seen very easily that the union of the paired vasa deferentia is merely external and not internal, since the lumina, or internal cavities, of the two ducts remain quite distinct.

The common vas deferens runs to a set of glandular structures which I regard as corresponding to a prostate gland, and consisting altogether of four blind tubular diverticula; a median pair of short tubules, which may be termed the median prostates (r.m.p.), and a much longer pair of lateral tubules, which may be called the lateral prostates (r.l.p.). The two median prostates are in close contact, but their cavities are quite distinct and independent. The walls of the tubules are composed of a single layer of glandular epithelial cells of small size, which show in surface view very distinct polygonal outlines (Pl. 30, B). The tubules contain

* The structure of the testis was not quite correctly described in our monograph on the development of *T. lewisi* (Minchin and Thomson, *l.c.*). When that was written I had not seen the epididymis uncoiled, and regarded the dilated bladder-like portion of the testis as a vesicula seminalis.

a cavity, relatively spacious, in which I have never seen any spermatozoa; they cannot therefore be regarded as vesiculae seminales, but probably have a purely secretive function.

The common vas deferens runs towards the median prostates and then loops round them in a peculiar manner, running in the valley between the two contiguous median prostates. Just after the two still separate ducts, which form by their apposition the common vas deferens, have passed the prostates, there is a slight dilatation of the ducts into which the prostatic tubules open, but quite separately; that is to say, the left median and left lateral prostate open into the left half, the right median and right lateral prostate into the right half, of the common vas deferens.

The common vas deferens, after receiving the openings of the prostates, runs on towards the penis as a duct which may be termed, as in other animals, the ductus ejaculatorius (d.ej.). Like the common vas deferens, however, the ductus ejaculatorius is a double-barrelled structure, consisting of two ducts in close contiguity, but with distinct internal cavities.

At the point where the ductus ejaculatorius enters the penis there is a most singular complication of structure. The proximal end of the penis is prolonged into a spirally coiled organ which, for lack of a better name,* I propose to call the "corkscrew-organ," since it resembles in form a corkscrew, or a spiral drill or borer, of about four turns (c.s.o.). The ductus ejaculatorius runs straight to the base of the corkscrew and through its axis; at the point where it enters the axis of the corkscrew the ductus ejaculatorius can be seen very plainly to be still double; it is difficult to make out clearly what happens in the axis of the corkscrew, but when this structure is viewed from the top, it is seen equally plainly that the duct emerges from the axis as a single duct, no longer double-barrelled. It is evident, therefore, that the two ducts that come from the testes, maintaining their individuality and distinctness up to this point, become confluent at some spot in the axis of the screw. This single duct, the duct

* I regret to say that my meagre acquaintance, which I have not had the leisure to extend, with the vast and scattered literature relating to the anatomical structure of insects, is inadequate to permit me to state whether this or similar organs in other insects have been studied in detail and whether there exists already a special technical term for the structure which I term here in a purely descriptive manner "corkscrew-organ."

which runs the whole length of the penis, may be called distinctively the urethral duct (d.).* The exact point at which it begins in the axis requires to be determined by sections.

The urethral duct emerges from the axis of the corkscrew at its apex and there turns and runs outwards round the outer edge of the spiral of the corkscrew, enclosed between two chitinated bars, or more correctly thickenings of the wall of the duct. These chitinous thickenings are best seen in unstained preparations of the penis (Pl. 29). The chitin on the inner side of the duct (*i.e.* on the side of it turned towards the axis of the spiral) is the thicker and stronger of the two, but is only continued from the base over about three turns of the spiral, while the thinner chitinous bar on the outer side of the duct is continued for nearly a whole turn more.

The structure of the corkscrew-organ is difficult to make out in full detail without sections, but if a portion of the spiral be carefully examined, the following points can be seen in dissections of the whole apparatus stained and mounted (Pl. 30, A). At the extreme outer edge of the spiral is seen the narrow urethral duct (d.) with its chitinous thickenings on the inner and outer side. Running from the axial region, which can also be seen in the unstained preparations to have a chitinous support (ax.), is a superficial layer of radiating striated muscles, which run across from the axis centrally to the duct peripherally; this layer can be focused without difficulty. At a deeper focus, below the radiating muscles, two structures can be made out lying between the axis and the duct; close to the axis and apparently attached to it, is a spiral muscle (sp.m.) composed also of striated muscular fibres; and between the spiral muscle and the urethral duct is a cushion of cells which appear to be glandular in appearance, but sections would be necessary to determine their precise histological nature. These various structures can be seen best in the lowest coil of the corkscrew; they are depicted in Plate 30, A, but it is difficult to

* If a dissection of the male reproductive organs be treated with caustic potash, everything up to the base of the corkscrew, that is to say the vasa deferentia, prostates and ductus ejaculatorius, dissolve away, but the urethral duct issuing from the apex of the corkscrew remains very distinct; and this is, as a matter of fact, the best way to study its course. It would appear, therefore, as if the urethral duct is distinguished from the other ducts by the possession of a chitinous lining, and therefore represents, probably, an ingrowth of the outer integument in origin.

combine clearly in one sketch things seen in the microscopic preparation at different foci.

Seen in life, that is to say in a freshly dissected flea, the corkscrew-organ is usually performing peculiar pulsating movements, which remind one to some extent of the movements of the hairspring of a watch, with the difference that the hairspring lies in one plane, while in the organ of the flea the axis of the spiral is prolonged vertically so that a form like a corkscrew results. It is seen that in the living condition the corkscrew becomes alternately first longer and narrower and then shorter and broader. The elongation and narrowing of the corkscrew is doubtless brought about by the contraction of the radiating superficial muscles; these in their turn are antagonised by the spiral muscle, which by its contraction would tend naturally to make the corkscrew shorter and broader.

As to the function of corkscrew-organ, I can only offer the suggestion that it may act as a sort of sperm-pump. The movements seen in the freshly dissected flea may perhaps become more active and regularly rhythmical during the act of copulation, and serve to pump the sperm on from the ductus ejaculatorius and vasa deferentia into the penis. This is, of course, a mere conjecture from the observed facts of its structure and activity. If, on the other hand, the cushion of cells between the spiral muscle and the duct be glandular in nature, the organ as a whole must have other functions in addition to that of acting as a pump.

The penis is an organ of complicated structure, which I will deal with briefly; Pl. 29 shows what I have been able to make out in preparations mounted unstained, or further cleared with potash before mounting. The penis (P.), which is very large in proportion to the size of the insect, is made up of strong thick bars of chitin. It is worked mainly by strong protractor and retractor muscles attached to a broad bar of chitin (b^1), which is a prolongation of the dorsal integument at the right and left margins of the pygidium. The median retractor muscles (m.r.) are attached distally to a prolongation of the dorsal side of the penis, and the lateral retractors (l.r.m.) are attached to a bar (b^2) which arises from the ventral side of the penis; there are two such bars, right and left, diverging from one another like a V; it is clearly impossible that the penis could be protruded farther from

the body than the point of insertion of these bars (b^2). I think it probable that there are more muscles attached to b^2 than are seen in my figure, but have become torn away in the dissection. Both b^1 and b^2 can be seen clearly through the body-wall in the uninjured flea. From the thick beam of chitin which forms the dorsal part of the penis a lateral muscle (l.m.) comes off, which is probably attached distally to the integument.

In one of my preparations treated with potash, spermatozoa could be seen very plainly in the interior of the urethral duct, and they have been put into the figure on Pl. 29 in order to show the course of the duct. The spermatozoa (sp.z.) begin in the lowest coil of the corkscrew-organ, where they show a peculiar festoon-like arrangement. As the duct passes into the body of the penis, the spermatozoa take on an arrangement in wavy bundles and the calibre of the duct widens considerably, and at the same time the spermatozoa show that the duct crosses over the chitinous bar which forms the inner boundary of the duct in the corkscrew-organ, and which has now become very much thinner and more delicate, passing on to be merged into a much thicker bar on the ventral side of the penis. Just a little in front of the middle region of the penis the spermatozoa are heaped up in a way that shows the duct to have become greatly enlarged in calibre, but behind this point the spermatozoa disappear altogether. In stained preparations it can be seen that the penis has a superficial layer of muscles which appear to have a criss-cross arrangement, and lie on the wall of the widened spermatid duct, but they have not been put into the drawing, as their exact position and arrangement are difficult to make out clearly. The contraction of the superficial muscles would doubtless have the effect of contracting the lumen of the penis and ejecting the sperm.

Such are the main points of the structure of this very complicated apparatus, so far as I have been able to make them out; but I think it probable that there are more minutiae to be described, especially with regard to the structural details of the penis and "corkscrew-organ."

IV. *The Female Reproductive System.*

As regards the primary sexual organs of the female sex, they are of the usual insectan type, and can be dealt with briefly.

There is a pair of ovaries, lying symmetrically right and left in the abdomen dorsal to the stomach. Each ovary consists of a number of ovarian tubes or ovarioles; usually four on each side, but in one of my mounted preparations there are five ovarioles in each ovary. The ovarioles are of the simplest type, composed of successive egg-chambers, increasing progressively in size, without special yolk-chambers. The ovarioles of each side unite into a short paired oviduct, and the paired oviducts of the two sides unite into a median unpaired oviduct, in which, probably, the ovum is fertilised and subsequently becomes invested by a shell.

In addition to the ovaries and oviducts, which are very easy to dissect out, there lies, ventral to the rectum, an organ found in all fertile female insects, the receptaculum seminis, into which the sperm is received at copulation and stored up in order to fertilise the eggs as required. The receptaculum and its duct are by no means difficult to dissect out and mount, and make a singularly beautiful and fascinating microscopic preparation (Pl. 31). The duct is coiled up into a veritable labyrinth, and the sole difficulty in the dissection is to uncoil it without breaking it.

The receptaculum itself (R.S.) is a chitinous capsule with a brown, delicately sculptured, semi-transparent wall, and a peculiar form. The main portion of the capsule, that portion from which the duct arises, is roughly spherical in form. At one point, which is distant from the origin of the duct by about one-third of the circumference of the main chamber, an outgrowth or diverticulum arises, forming a second chamber, which is horn-shaped, and bends round the main chamber. The horn-shaped chamber is connected, on its concave side, to the main chamber by a sheet of striated muscle (m.r.s.). The contraction of these muscle-fibres must clearly have the effect of approximating the horn-shaped chamber to the main chamber, and at the point where the horn-shaped chamber arises from the main chamber there is a rim of chitin which appears to be softer than the rest of the wall, forming a weaker spot which apparently serves as a hinge, allowing the horn-shaped chamber to be moved slightly; it is at this spot that artificial deformations of the wall of the capsule are often caused as the result of slight shrinkage when the receptaculum is mounted in Canada balsam. The receptaculum is usually packed with spermatozoa, which can be seen through the wall of the capsule, but better still if the capsule be burst

open by pressure under a cover-glass when freshly dissected out. In one of my specimens the receptaculum is empty and contains no spermatozoa; this specimen is also the only one I have succeeded in mounting in Canada balsam without any shrinkage taking place in the hinge-region. This virgin receptaculum also shows some structures in the interior, the nature of which I have not been able to make out clearly, but which look rather like prolongations of the duct into the interior of the main chamber. Sections would be necessary, however, to determine the nature of these internal arrangements, which are not visible in any of my specimens that are filled with spermatozoa.

The duct of the receptaculum (d., d., d.) is of extraordinary length, and just where it arises from the main chamber it is surrounded by a cushion of deeply staining, closely packed cells of glandular appearance (gl.c.), each shaped somewhat like an Indian club. The duct itself has an internal chitinous lining secreted by an external epithelial layer, which is shallow and contains small nuclei in great number but shows no distinct cell-outlines. At its proximal end, immediately after it comes through the glandular cushion already mentioned, the duct is surrounded by a great number of rounded cells (gl.), which have clear, lightly staining contents, and present also a glandular appearance. The rounded cells are thickly clustered round the proximal end of the duct, but as the duct is followed along in a distal direction they diminish in number and gradually thin out until, about half-way along the duct, they disappear altogether, and the distal half of the duct consists only of the chitinous lining and the epithelium with small nuclei.

As the duct approaches its termination it shows some peculiar complications, forming what I propose to call the terminal organ (T.O.). First of all there is a feeble imitation of the corkscrew-organ in the shape of a broad expanded plate, apparently chitinous, on one side of the duct, which performs a spiral twist of one complete turn. The spiral portion passes on into a short length of the duct, which has on one side a thickening of the chitin to form a strong bar (c.b.), bent like a bow, which is strung, so to speak, by a strong muscle of four or five fibres (m.t.o.). I have not been able to determine exactly by dissection where the duct finally opens, whether into the unpaired oviduct or into a terminal genital vestibule or vulva; sections, or

perhaps specimens cleared in potash, would be necessary to determine this point.

The spermatozoa live a very long time, as is well known, in the receptaculum, and are used up gradually to fertilise the eggs as they are laid. In the queen-bee it is known that the insect lays fertile eggs for at least three years, and in some other insects this length of time may be exceeded by a considerable amount. The muscles seen in connection with the receptaculum and its duct may be connected with the function of passing out the spermatozoa. A contraction of the muscle connecting the horn-shaped chamber of the receptaculum with the main chamber would probably force some spermatozoa out into the duct. On the other hand, a contraction of the "bowstring" muscle of the terminal organ would bend the "bow," and so occlude the duct, preventing anything from passing out. There does not seem to be any apparatus for forcing the spermatozoa up the duct and into the receptaculum, but this is effected probably during copulation by the male intromittent organ—possibly by the problematic "corkscrew-organ." The spermatozoa in the receptaculum must be kept alive a long time, and may be nourished by the secretion of the glandular cushion round the origin of the duct, while the rounded gland-cells on the duct may perform some similar function for the spermatozoa during their passage down the labyrinthine duct, the great length of which is difficult to explain in a plausible manner. All these suggestions have, however, only the value of more or less probable surmises.

In the tsetse-fly the receptaculum is a paired organ, and in the gnat there are three receptacula, one median and two paired. In that of the flea there is no sign of any double structure.

V. *Muscle-cells of Stellate Form in the Oesophagus of the Flea.*

In some of our smear-preparations of teased flea-stomachs, made in the course of our investigation into the development of *Trypanosoma lewisi*, there were to be found occasionally specimens of the flea's oesophagus, which adhered to the cover-glass after it had been fixed with Mayer's sublimate-alcohol mixture and stained by Heidenhain's iron-haematoxylin method. In such preparations it is easily seen that the oesophagus has a beautiful

and very delicate layer of muscular tissue, in the form of a network (Pl. 32, A, B and C). The individual muscle-cells are branched like ganglion-cells, and their processes anastomose to form the network. Some nodes of the network are formed merely by the union of two or three such processes, while other nodes are formed by the body of the cell, and contain the cytoplasmic cell-body with a nucleus. The processes themselves are transversely striated, and form the actual muscle-fibres. It can be seen that at a cellular node of the network the striated fibres pass right through the body of the cell and come out on the other side, their striation and individuality becoming slightly less distinct in their passage through the cytoplasm of the cell.

Remembering that I had seen muscle-cells of a somewhat similar type in the "crop" or "sucking stomach" of the tsetse-fly, an organ which is morphologically a diverticulum of the oesophagus, I made a preparation of the crop of a common house-fly and found a musculature of a very similar type (Pl. 32, D, E). The main differences are, first, that the cells are on a much larger scale of size, requiring lower powers of the microscope for their study; secondly, that the muscular network has a definitely rectangular arrangement, those fibres which run in certain directions being considerably thickened, and connected with one another by delicate fibres running across at right angles. In some parts the thickening of these longitudinal fibres is much more marked than in others. All the fibres, even the thinnest, show the characteristic transverse striation very distinctly.

The resemblance of the muscular network in the two cases raises some interesting points of phylogeny. In the first place, it should be noted that a contractile network is the most efficient arrangement for the contraction of a bladder, since it gives an even contraction in all directions. The muscles of the human urinary bladder are also arranged in a network, but on a much larger scale than those described here, since the strands of the network are not outgrowths of individual cells, but are made up of thick bundles of contractile cells. It is therefore not surprising to find a network in the contractile elements of an organ such as the crop of the house-fly. On the other hand, it is rather remarkable to find it in the oesophagus of the flea.

Entomologists are generally agreed in regarding the fleas as modified and specialised Diptera—that is to say, as descended from fly-ancestors. If so, they may have once possessed a crop such as is found in the fly, but which, with reduction in the size of the body, has gradually disappeared, and has ceased to be developed. Since, as has been pointed out, the crop is formed as a diverticulum of the oesophagus, the existence of such an organ in the ancestors of fleas might explain the persistence of a musculature of this peculiar type in the oesophagus of the flea. But it would be necessary to examine the oesophageal musculature of other insects before adopting this theory as an explanation of the presence of stellate muscle-cells in the flea.

DESCRIPTION OF PLATES.

PLATE 26.

ABDOMINAL NERVOUS SYSTEMS OF THE MALE (LEFT) AND FEMALE (RIGHT) FLEA, MAGNIFIED 90 DIAMETERS.

th.³, metathoracic ganglion; abd.¹, abd.³, abd.⁵, and abd.⁶, first, third, fifth, and sixth abdominal ganglia; abd.⁷, 7th abdominal ganglion, present in the male, wanting in the female; T.g., terminal ganglion-complex. Note the difference in the size and number of the nerves that arise from T.g. in each case.

PLATE 27.

SALIVARY GLANDS OF THE LARVAL AND ADULT FLEA.

A, salivary gland of the larva, magnified 60 linear; d., duct, showing at its distal extremity (to the right) the union with the corresponding duct from the other side of the body; a small portion of the duct is seen magnified 400 linear; r., reservoir; l.a.¹ and l.a.², the two anterior lobes of the gland; l.p., the posterior lobe.

B and C, the salivary glands of the adult flea, at B magnified 60 diameters, for comparison with A, at C magnified 160; d., duct; gl., the two pouch-like glands.

PLATE 28.

GENERAL VIEW OF THE REPRODUCTIVE APPARATUS OF THE
MALE RAT-FLEA, SEEN FROM THE RIGHT SIDE.

T, T, the two testes; ep.¹, the left epididymis in its natural coil; ep.², the right epididymis forcibly uncoiled; v.d.¹, v.d.², the left and right paired vasa deferentia; v.d.³, the common vas deferens; r.l.p., the right lateral prostate gland; r.m.p., the right median prostate; d.e.j., the ductus ejaculatorius; c.s.o., the "corkscrew-organ"; d., the urethral duct running spirally round the corkscrew-organ; P, the penis. $\times 75$ linear.

PLATE 29.

CHITINOUS SKELETON AND PRINCIPAL MUSCLES OF THE PENIS AND
CORKSCREW-ORGAN OF THE MALE RAT-FLEA, FROM THE LEFT
SIDE.

P, penis; e.p., external plates of the posterior end of the body; b.¹, chitinous bar, an outgrowth of the dorsal integument; b.², chitinous bar arising from the ventral-posterior end of the penis; l.r.m., lateral retractor muscles running from b.¹ to b.²; m.r.m., median retractor muscle running from b.¹ to an outgrowth of the dorsal side of the penis; l.m., lateral muscle running from the side of the penis to the body-wall; p.m., protractor muscle, running from b.¹ to the penis; c.s.o., corkscrew-organ; ax., its axial skeleton; d., the urethral duct; sp.z., sp.z., spermatozoa in the duct, indicating its course where the corkscrew-organ passes into the penis. $\times 150$ linear.

PLATE 30.

DETAILS OF THE MALE REPRODUCTIVE SYSTEM MORE HIGHLY
MAGNIFIED.

A, a portion of one of the coils of the "corkscrew-organ," seen from the lower surface; d., the urethral duct, running at the extreme outer edge of the spiral; ax., the chitinised axis of the spiral; running from ax. to d. are the superficial radiating muscles, clearly seen; below the radiating muscles two structures are

seen, less clearly; close to ax. is a spiral muscle, sp.m.; between sp.m. and d. is a cushion of cells with nuclei, apparently glandular in nature. $\times 350$.

B, a portion of one of the prostatic tubules, in surface view, showing an epithelium composed of glandular cells with very distinct polygonal outlines. $\times 350$.

PLATE 31.

ACCESSORY REPRODUCTIVE APPARATUS OF THE FEMALE RAT-FLEA.

R.S., receptaculum seminis; m.r.s., muscles connecting the main chamber of the receptaculum with its horn-shaped prolongation; gl.c., cushion of gland-cells surrounding the duct at the point where it issues from the receptaculum; d., d., d., the long coiled duct of the receptaculum, seen here forcibly uncoiled; gl., gland-cells, thickly crowded on the proximal part of the duct; T.O., terminal organ; c.b., bar of thickened chitin on the wall of the termination of the duct; m.t.o., "bowstring" muscle of the terminal organ. The apparatus is seen at a magnification of 200 linear; at A and B are seen portions of the duct magnified 400 linear.

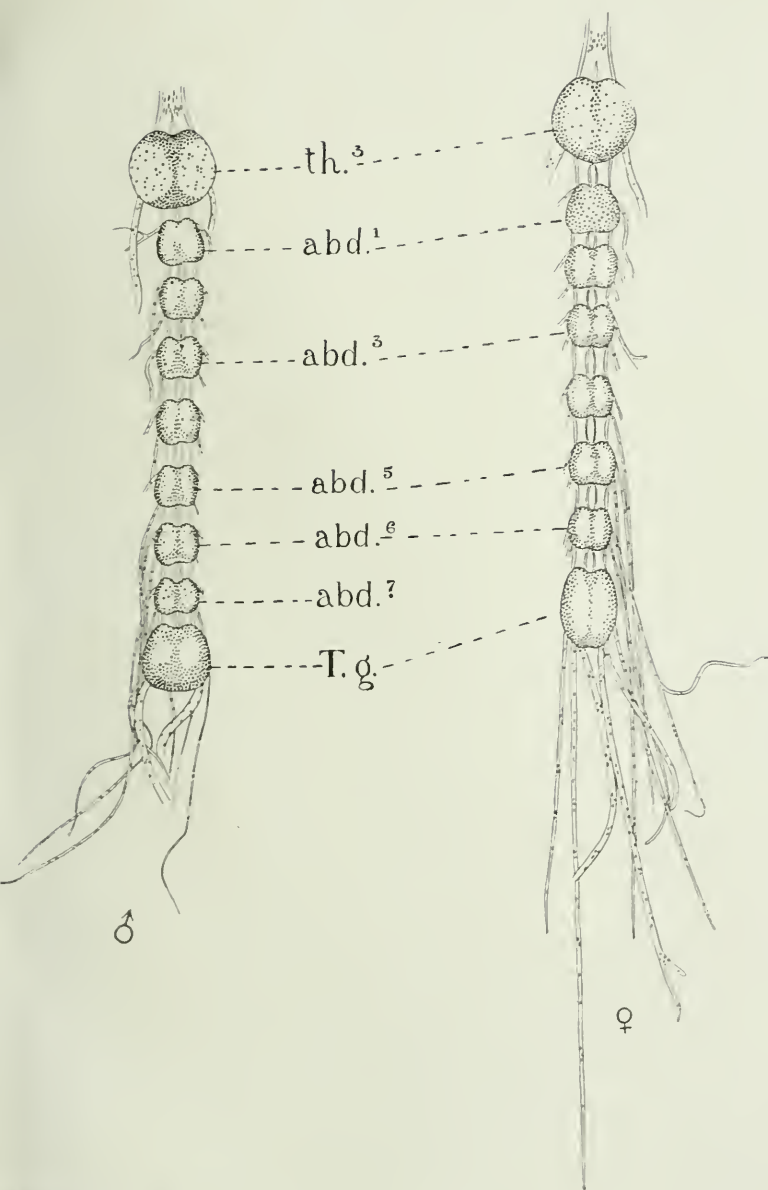
PLATE 32.

STELLATE MUSCLE-CELLS OF THE OESOPHAGUS OF THE FLEA AND THE CROP OF THE HOUSE-FLY.

A, oesophagus of the flea, magnified $\frac{1000}{3}$ linear, showing the muscular network; B, a detail of A, magnified 1,000; C, a detail from another specimen, magnified 1,000, showing how the striated muscle-fibres are continued through the cytoplasm of the muscle-cells.

D and E, muscular network from two different regions of the crop of the house-fly; both from the same specimen, and magnified to the same degree as A.

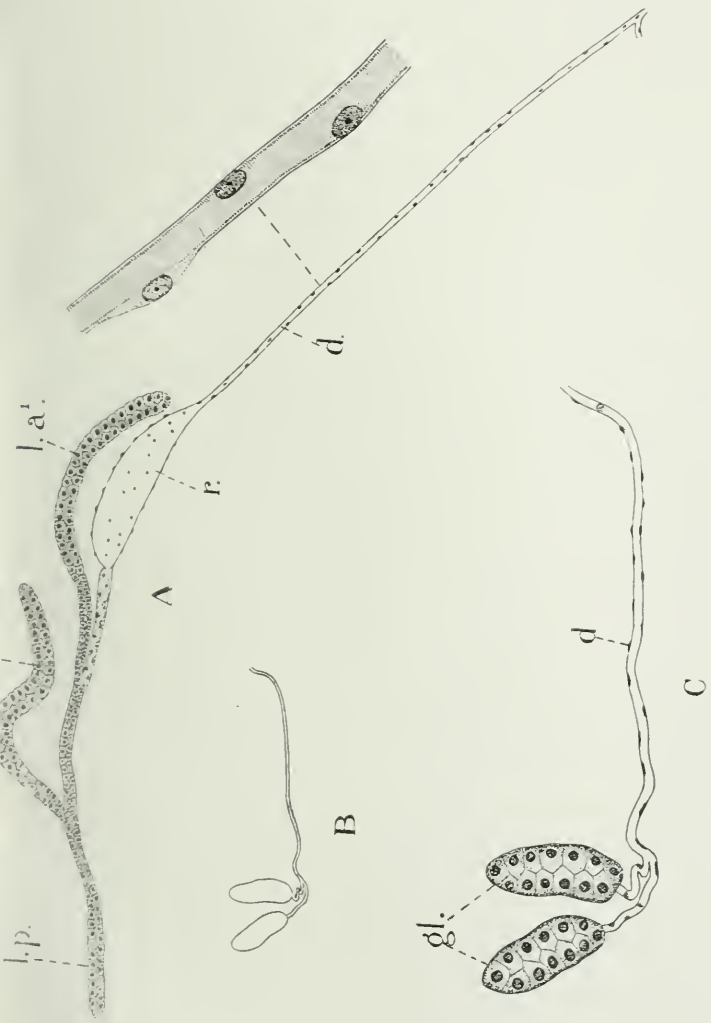
LISTER INSTITUTE,
January 25th, 1915.



MABEL RHODES, *del. ad nat.*

ANATOMY OF THE RAT-FLEA.

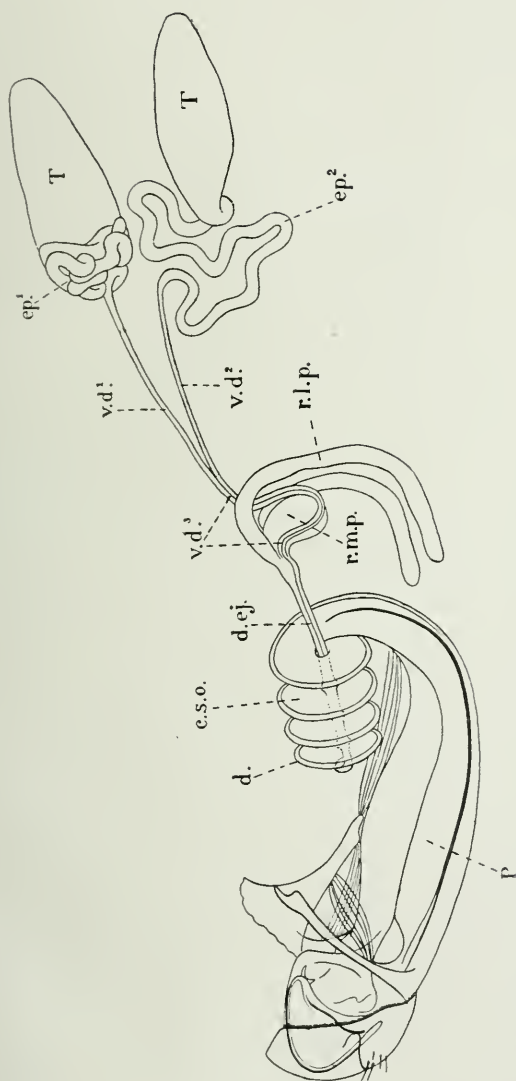




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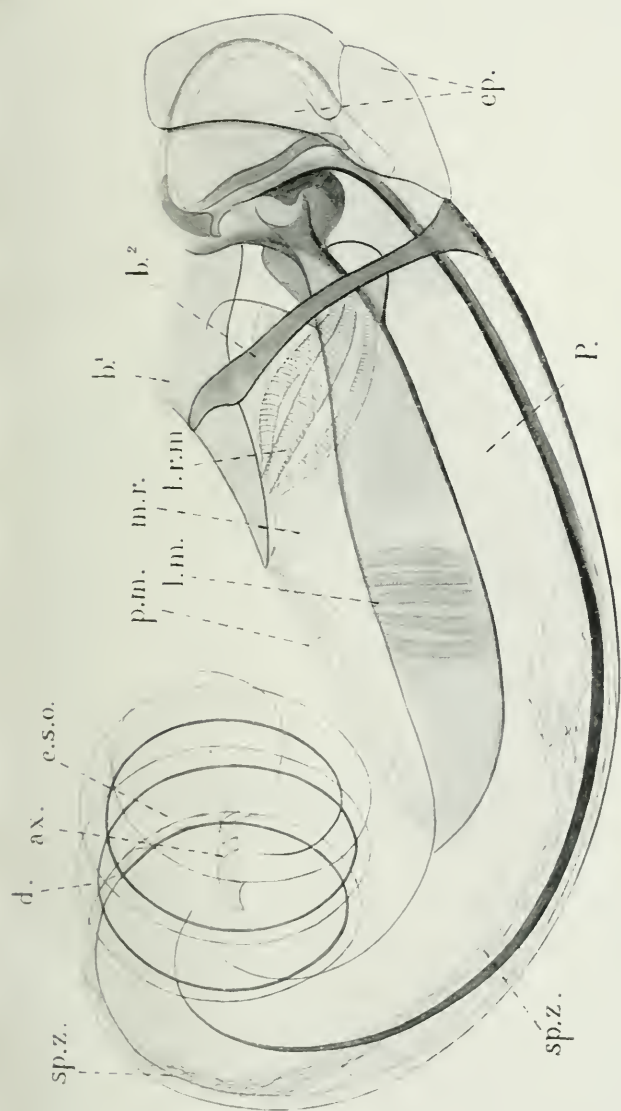
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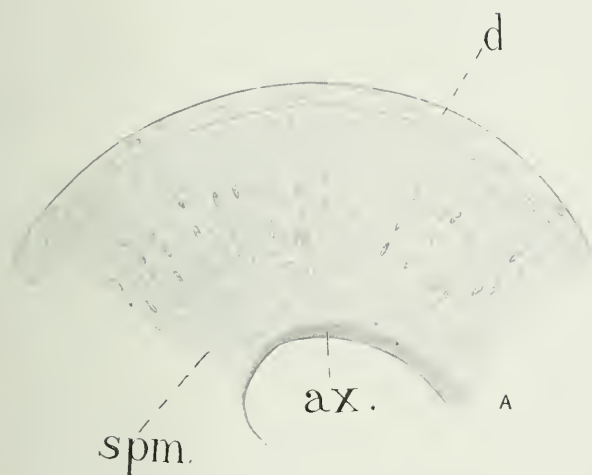
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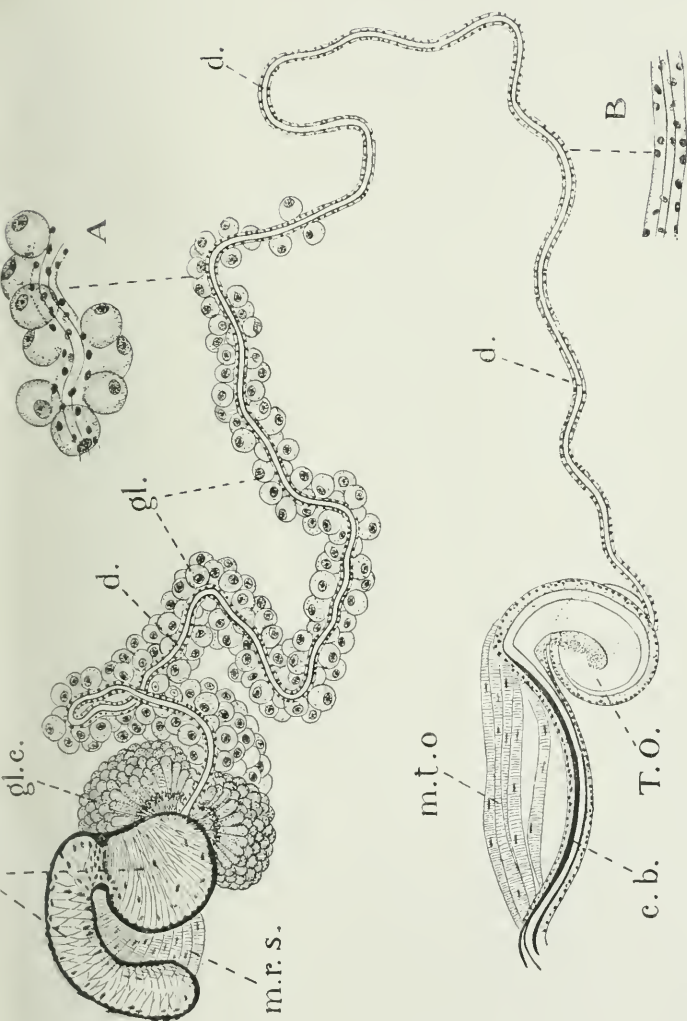
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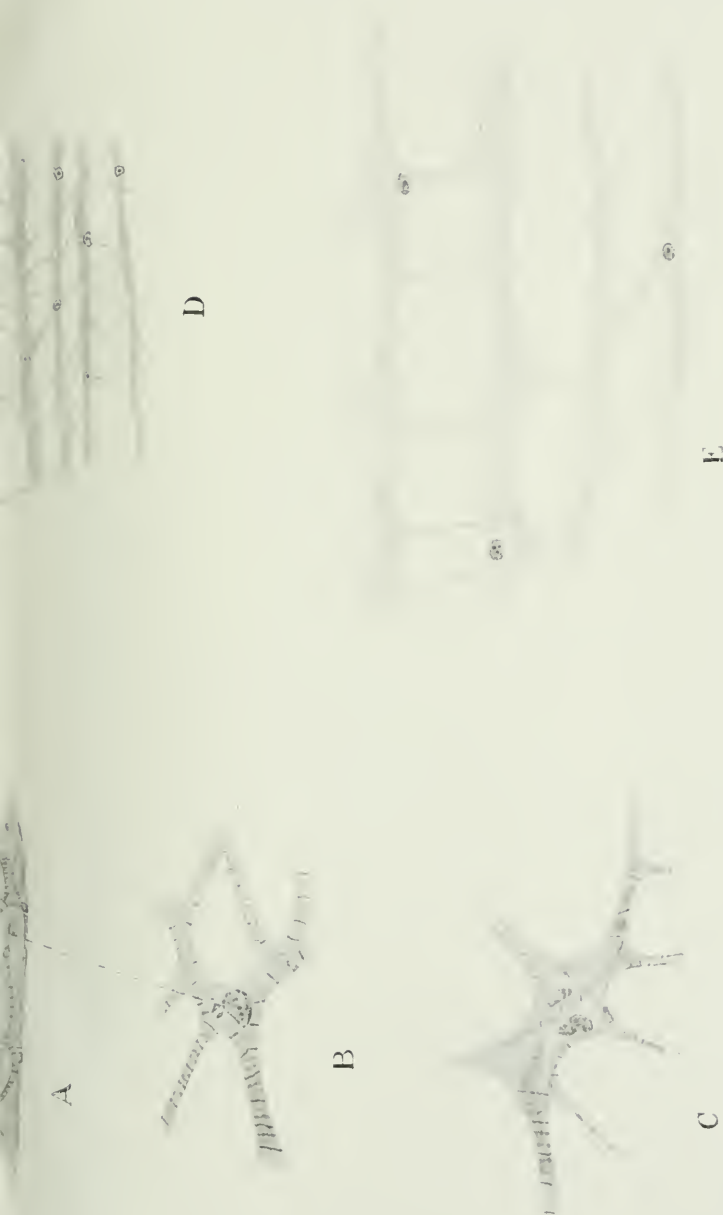
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ANATOMY OF THE RAT-FLEA.

MAHEL RHODES, *del. ad nat.*

The Rat-Trypanosome, *Trypanosoma lewisi*,
in its Relation to the Rat-Flea, *Ceratophyllus fasciatus*.

By

E. A. Minchin

and

J. D. Thomson.

With Plates 36—45 and 24 Text-figures.

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PART I. INTRODUCTORY.

(1) PERSONAL NARRATIVE.

IN this memoir we give a detailed account of investigations which have occupied us intermittently, with many interruptions, during the past five years. When we undertook this task our object in view was to work out as fully as possible the life-history and mode of transmission of a trypanosome, so that in at least one species of these important parasites its relation to the invertebrate host might be as thoroughly known as, for instance, the relation of the malarial parasite to the mosquito, thus furnishing a standard with which the life-histories of other species of

trypanosomes might be compared and contrasted as they become known. How far we have succeeded in our task must be left to our readers to judge.

The species which we selected as the subject of our investigations was *Trypanosoma lewisi*, the common parasite of rats which is apparently of world-wide distribution. This species offers many advantages for such a study. It is common in London and easily procured when required; its vertebrate host, the rat, is a mammal of small size, the domesticated variety of which lives well and breeds rapidly in confinement, is inoffensive, and is easily handled; its invertebrate host, the rat-flea, is also easy to keep in captivity and is extremely prolific, and it is of a size which, though it increases to some extent the difficulties of manipulation, has a great advantage that the material to be searched and studied microscopically is confined within a small compass¹; and finally, by no means the least of the advantages of working with *T. lewisi* is the fact that it is non-pathogenic to its natural host and cannot live at all in human blood.

Since there is no difficulty whatever in obtaining the vertebrate host in abundance, either in the clean (i. e. non-infected) or infected condition, our first care was to obtain a stock of the invertebrate host, the flea. This we succeeded in doing from rats trapped in the open near Mr. Gurney's Laboratory at Sutton Broad, Norfolk. Fifty specimens of *Ceratophyllus fasciatus* were obtained in this way in the autumn of 1908, and were kindly identified for us by the Hon. N. C. Rothschild, and with these fleas a breeding-cage was stocked and a flea-farm started. The cages used were of the type used by the Plague Commission, as figured in the 'Journal of Hygiene,' vol. vi, pl. iv. A rat was kept in the cage to feed the fleas, and they were left to themselves. Early in 1909 one of us (E. A. M.) went to Rovigno for some

¹ An advantage which those will appreciate who have had practical experience of searching for trypanosomes through many centimeters of the digestive tract of the tsetse-fly, for instance.

three months, during which time the fleas were left to breed under the care of an assistant, whose duties consisted of attending to the rat and replacing it if it fell ill or died. When the cage was examined after Easter it was found to be swarming with fleas, and our work began in May, 1909. We have worked ever since then with the fertile progeny of the original fifty fleas from Norfolk, and have never added further to our stock from without. The fleas breed so fast that it is often necessary to keep their numbers down, otherwise they take too much blood from the rat and affect its health. Fresh breeding-cages have also been started, and during the greater part of the time that we have been at work we have kept two cages constantly going, one in which the fleas are fed always on a clean healthy rat, and another in which an infected rat is always kept. We shall refer to these two cages as the non-infected and the infected breeding-cages respectively. As will be shown below, the stock of fleas with which we have worked all along was fortunately quite free from any natural infection with leptomonaad or other flagellate parasites. Thus we have been saved from a fertile source of confusion and error, since we can be quite certain that any flagellates found in our fleas are stages of *T. lewisi* and nothing else.

Although we cannot claim that in our work we have solved completely every problem presented by the transmission of the trypanosome and its development in the flea—a result which probably no man could achieve in a life-time—we think it now fitting that we should publish such results as we have obtained, after having done as much as we were able to do in the time and under the circumstances. We claim at least that we have not jumped to our conclusions; our note-books contain not only the records of many experiments, but also of the dissection and examination of over 1,600 fleas, and we have over 700 drawings of stages of the development of the trypanosome, from which those given in this memoir are a selection. It would, indeed, have been easier for us to have written a plausible and apparently complete account of

the development of *T. lewisi*, full of positive statements, after one year of our work than it is after five years, during which we have been forced by the logic of facts to abandon or modify many of our earlier conclusions or beliefs.

It is our pleasant duty at this point to express our thanks to those of our friends and colleagues to whom we are indebted for assistance. To Dr. Woodcock and Miss Robertson we are grateful for much advice, friendly criticism, and valuable suggestions. Our work could not have been carried out, certainly not in the time at all events, without the assistance of Miss Rhodes, who has not only drawn all the illustrations with a skill to which it is quite unnecessary for us to draw the reader's attention (since the figures speak for themselves), but has also relieved us of a large part of the wearisome drudgery of searching through the microscopic preparations. Mr. George Kauffmann has been most helpful in every part of the investigation, not only assisting in making preparations, examining rats, and other similar duties, but more especially in carrying out intelligently and enthusiastically all the details of the experiments, in which his extraordinary skill and resourcefulness in controlling the wayward flea were invaluable. Dr. D. J. Reid has given us the benefit of his skill and experience in microphotography. From our colleagues of the Lister Institute, Dr. C. J. Martin, the Director, and Mr. Bacot, who have been themselves engaged in studying the transmission of plague by fleas, we have had many valuable hints and help in various ways. To each and all of these we desire to express our cordial thanks and gratitude.

(2) NOTES ON THE FLEA, CERATOPHYLLUS FASCIATUS.

(a) Anatomy. Methods of Dissection.

The fleas collected for dissection and examination were thrown, or allowed to hop, on to the surface of a small

quantity of salt-citrate solution¹ placed in a suitable glass capsule. The fleas are quite helpless on the surface of the liquid, and each flea that it is required to dissect can be picked off the surface of the liquid and transferred to a small drop of the same solution on a slide for further operation.

The examinations of the fleas were usually conducted by both of us acting in concert. One of us worked with the dissecting-microscope, extracted the parts of the flea required, placed them on slides, covered them with glass slips, and handed them to the other, who proceeded to search them carefully through under a microscope, using dry lenses of fairly high magnification (Zeiss D or apochromatic 4 mm.). In some cases one of us worked entirely alone, but it is difficult for one person to carry out satisfactorily both the dissection and examination of the flea; the various parts of the digestive tract often require prolonged and careful searching to find the flagellates, and if the operator be working single-handed, one preparation may dry up while he is searching through another.

For the dissection of the flea² the following apparatus was used: A pair of fine needles mounted in wooden handles, a fine pair of forceps, and a dissecting-microscope, besides slides and coverslips. The needles used were sharpened on a hone, one to a sharp point, the other to a flat, chisel-like edge with rounded corners. The pointed needle was the more useful for holding, the flat-edged needle

¹ Made up as recommended by Laveran and Mesnil, namely: 1 grm. of sodium citrate and 1 grm. of sodium chloride dissolved in 200 cc. of distilled water. This mixture appears to be most favourable for the examination of living trypanosomes.

² If an operation can be properly called dissection which consists in treating the flea as the Thracian women are said to have treated Orpheus: "*Discerptum latos juvenem sparsere per agros*" (i. e. fields of the microscope, in this case). It need hardly be said that our object was not to study the anatomy of the flea, but to extract from its body those organs which might possibly harbour developmental stages of the trypanosome.

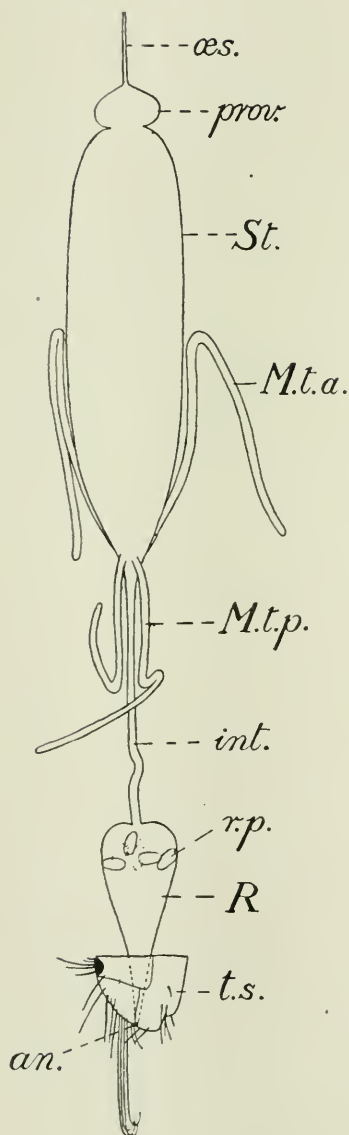
for cutting. The dissecting-microscope used was a Zeiss binocular, with No. 4 eyepieces and the paired objectives F⁵⁵. It is also necessary for the dissector to have at hand an ordinary microscope armed with a low power, since it is often difficult to distinguish the minute organs of the flea under the dissecting-microscope; the intestine, for instance, if severed from its connections might easily be confounded with a portion of a Malpighian tubule.

In the following paragraphs is described the method of procedure for making what may be considered an exhaustive examination of a flea for trypanosomes; it was not always necessary, however, to attempt so much, nor is it claimed that the entire operation was always successfully carried out, since both our knowledge of the flea's anatomy, and our skill in extracting the organs required, advanced considerably during the progress of our investigations.

The flea, as stated above, is picked up with a fine pair of forceps, holding it by its head, and placed on a slide (slide 1) in a drop of salt-citrate solution. The first operation is to cut off the head, which is not always easy if the flea be a lively one, in which case it is best to asphyxiate or drown the flea partially by holding it under water with the forceps for a short time. To decapitate the flea, hold it still by pressing the pointed needle across the thorax, and with the flat-edged needle cut across the head in the region of the eyes. The severed head may then be removed to another slide (slide 2), covered with a cover-glass, and the contents of the proboscis examined; but as the proboscis was never found to contain trypanosomes we ceased to trouble about it in our later studies.

It is frequently the case that the flea has its rectum filled with fæces or with partially digested blood, and when this is so it happens commonly that the rectum empties itself by a violent contraction at the instant that the head is severed (sometimes also eggs are extruded); or if the evacuation does not take place at this point in the proceedings, it is very difficult to avoid squeezing out the contents of the distended

TEXT-FIG. 1.



Digestive tract of a female flea, dissected out and drawn with the camera lucida at a magnification of 60, reduced in the reproduction to 40. The anterior part of the dissection is seen in ventral view; the rectum and its surroundings in side view. *æs.* Œsophagus. *prov.* Proventriculus. *St.* Stomach. *Mt.a.* Malpighian tubule of the anterior pair; that on the left side of the stomach is shown in its normal position, that on the right has its distal limb pulled out and away from the stomach. *Mt.p.* Malpighian tubule of the posterior pair. *int.* Intestine. *r.p.* The six rectal papillæ. *R.* The rectum. *t.s.* Terminal segments. *an.* The anus.

rectum during the subsequent operation of opening the abdomen. In cases where fæces are thus extruded the body of the flea is removed at once to another slide (slide 3), and the fæces left on slide 1 are covered with a slip and examined.

Through the integument of the flea the stomach can be seen lying ventrally in the anterior $\frac{2}{3}$ of the abdomen, and often the rectum can be seen at the hinder end in the dorsal region. The ventral posterior and dorsal anterior part of the abdomen is seen to be occupied by a whitish mass, most conspicuous in the female, and consisting chiefly of the reproductive organs.

The next stage in the proceedings is to open the body of the flea. This is done near the hinder end, at about the level of the fourth or fifth tergite of the abdomen. The body is held still with the pointed needle, with which the thoracic region is pressed down or speared, and with the flat-edged needle the body-wall is cut through dorsally and ventrally in the region indicated, and the hindmost segments of the abdomen gently detached in such a way as to separate the integumental portions without rupturing or tearing the internal organs. It is especially important, if it be desired to examine the contents of the body-cavity, that the digestive tract should not be in any way torn or punctured. By holding the anterior part of the body and pulling gently on the detached hinder part, the gut can be stretched out and seen in nearly its full length; the stomach, usually containing a greater or less amount of more or less digested blood, is seen projecting from the anterior part of the body, the rectum is contained in the detached hinder part, and stretching between the two is the intestine like a delicate white filament, exposed in its whole length, but more or less obscured by the fat-body, Malpighian tubules, and generative organs, especially by the large ovaries in the female; these organs render the female flea much more difficult to dissect, in spite of its larger size, than the less-encumbered male. The generative organs and as much as possible of the fat-

body are now pulled out on to the slide and cut off from the body, care being taken not to injure the gut. The carcase of the flea, with the hinder part hanging on by the still intact intestine, is now removed to another slide (slide 4), and the extracted contents of the body-cavity on slide 3 can be covered with a slip and passed on for examination; but so far as stages of *T. lewisi* are concerned, it is superfluous to do so, since they are never found in the body-cavity unless the gut has been punctured or ruptured.

The next step is to divide the digestive tract into two parts, thereby severing completely the hinder part of the body from the fore-part. This is done at the point at which the Malpighian tubules are given off at the junction of the stomach and intestine, the region which represents the transition from the mid-gut, lined by endoderm, to the hind-gut or proctodæum, lined by ectoderm. The Malpighian tubules are four in number in the flea; two of them run forward a short way on the wall of the stomach right and left, attached to it by fine tracheal tubes, and then turn backwards again with a sharp, elbow-like bend towards the dorsal side of the body; the other two tubules run backwards parallel to the intestine and alongside of it towards the hinder end of the body. The posterior pair of the tubules are also bent on themselves towards their distal extremities, but not so regularly as the anterior pair. The gut is cut across with the flat-edged needle at the point of origin of the tubules, and if this be performed accurately one pair of tubules (the anterior pair) remains attached to the stomach, the other pair to the intestine; sometimes, however, all four tubules remain attached to one or other of these organs. The hinder part of the body, with the intestine and rectum, is now removed to another slide (slide 5). The stomach is then pulled backwards out of the anterior part of the body on slide 4, and with it come out also, continuous with its anterior termination, the proventriculus and the œsophagus, these two parts representing the embryonic stomodæum, lined by ectoderm, while the stomach represents the whole of the embryonic mid-

gut. The proventriculus is lined by a thick chitinous cuticle prolonged into stiff, curved, pointed spines, densely planted and forming, apparently, a straining apparatus; it is approximately globular in form and usually contains blood. The œsophagus is a delicate tube, its walls composed of the chitinous cuticle internally and a delicate network of muscles externally; it generally performs active movements, twisting from side to side, when freshly extracted.

The two pairs of salivary glands are situated in the anterior region of the abdomen right and left of the stomach. Each gland has the form of a simple oval pouch, the wall of which is composed of a single layer of large cells with very large nuclei. From each gland comes off a duct, which, after running a short distance, unites with the similar duct of the other gland of the same pair. (In one instance we have seen the two glands of one side of the body fused into one, but with their ducts quite separate; on the other side of the body there was a pair of distinct glands in their normal relations). The common duct of each pair of glands then passes forwards alongside the gut through the thorax into the head, where it meets and joins with the corresponding duct from the other side of the body. The common salivary duct then runs a short distance and opens into the proboscis, doubtless on the hypopharynx as in other insects. The salivary ducts are recognisable at once under the microscope by their trachea-like structure, being lined by a thick cuticle which has ring-like thickenings; the rings are, however, somewhat irregular and easily distinguishable from the very even and regular spiral thickening of the wall of a tracheal tubule. Externally to the cuticular lining the tubule is covered by an investing layer of protoplasm, of uneven thickness in different parts and containing fairly large nuclei at irregular intervals. The ring-like thickenings of the cuticular lining become less marked as the ducts approach their point of junction, and cease altogether before they unite; the cuticular lining being quite smooth in the common duct and for short distances in the paired ducts.

Not infrequently the salivary glands come out with the stomach when it is pulled out; more usually, however, they do not do so, but remain *in situ*. In such cases the anterior part of the body is removed to another slide (slide 6), and the stomach, left on slide 4, is teased up, covered, and handed on for examination.

Now the dissection of the hinder part of the body, on slide 5, is proceeded with, in order to extract and separate the intestine and rectum. The rectum, situated dorsally to the accessory reproductive apparatus, penis or receptaculum seminis, is a fairly large pear-shaped organ, the stalk of the pear terminating in the anus. The slender intestine joins the rectum at its broad end, and in this region are situated the six conspicuous rectal papillæ, remarkable and very characteristic structures, the presence of a single one of which makes it easy to recognise even a small fragment of the rectum. Behind the papillæ the rectum has a thin wall, to which the crithidial stage of the trypanosome, when present, is usually found attached, sometimes in vast numbers. In its anterior part, the region of the papillæ, the rectum has only circular muscle bands, between which are wide interspaces. In the hinder region, behind the papillæ, there are both circular and longitudinal muscle-bands; the latter can be traced forward to just behind the papillæ, at which point each band becomes rapidly narrowed to a tendon-like fibre, and at the same time the striations of the muscle disappear. The tendinous continuations can be traced forwards, in the living condition, for some distance, but we have not made out the exact points of their insertion.

The intestine is characterised by a continuous coat of ring-like muscle-bands, with interspaces, arranged very regularly external to the epithelium. When the edge of the intestine is focussed under the microscope, the layer of circularly-disposed muscle-fibres is seen in optical transverse section like a string of beads. The intestine is frequently seen to be performing active peristaltic movements, and it may be thicker in some parts than in others, owing to the contraction of the muscles.

The rectum must be dissected carefully out of the hinder part of the body, so that it remains on the slide, free from all the adjacent organs or chitinous plates of the integument. The easiest way to do this is to make an obliquely longitudinal cut with the flat-edged needle so as to sever the ventral-anterior half of the hindmost segments, together with the genitalia, from the dorsal-posterior half containing the rectum and anus. The genitalia can then be removed and the rectum extracted without much difficulty. It requires some care to separate it from the anus without injuring it. When this has been accomplished, all unnecessary débris is cleared away. If it be desired to make separate examinations of the intestine and rectum, the intestine is cut off as close as possible to its junction with the rectum. To effect this it is best to spear the rectum with the pointed needle and make the cut with the flat-edged needle; or the operation of cutting off the intestine may be performed before the rectum has been dissected out from the hinder part of the body. In either case, the intestine is removed to another slide (slide 7), and both rectum and intestine, on their respective slides (5 and 7) are teased up, covered, and passed on for examination. It is not difficult to tear the rectum into several pieces with the needles, but it is not so easy to tease up the intestine; it is too slender to make sure of splitting it lengthways, except by good luck and more or less accidentally, and it is necessary as a rule to content oneself with cutting it transversely into two or three short pieces, the contents of which are generally squeezed out during the process.

Finally there remain the salivary glands, on slide 6, in the portion of the carcase consisting of the thorax and fore-part of the abdomen from which the gut has been extracted. The salivary glands, as has been stated above, are lodged in the fore-part of the abdomen beside the stomach, and it is generally by no means difficult to extract them when the stomach has been removed. To do this it is best first to spear the thorax with the pointed needle, then insert the flat-edged needle into the abdominal cavity from behind, and rake

out gently the contents of the abdomen. The salivary glands sometimes come out fairly clean, but more often they are embedded in fat-body, tracheæ, etc., from which they must be carefully freed as much as possible. In such cases they are sometimes a little difficult to detect under the dissecting microscope, but their position may be traced by their long, thread-like ducts. They are much smaller in the male flea than in the female. Another method which sometimes succeeds better in extracting the glands is to pull on the integument of the thorax with one needle and on that of the abdomen with the other. The body-wall then often tears across at the junction of the thorax and abdomen, and the salivary ducts are seen at once stretched out between the two. By continuing to pull the thorax forwards, the glands may be pulled out of the abdominal cavity and are seen hanging on to the back of the thorax, from which it is not difficult to detach them. By this method the glands may often be obtained very clean and free from encumbering fat and other tissue. When the glands have been extracted, other débris is cleared away and the coverslip is put on. The glands are very soft and are crushed immediately by the weight of a coverslip if there is no other tissue under it; but for examination of their contents this is not a disadvantage.

In the foregoing paragraphs we have given a detailed account of a full examination of the flea, such as we practised in the earlier periods of our investigation. But when it became evident to us that the trypanosome, during its development in the flea, never strays beyond the limits of the digestive tract proper, we were able greatly to curtail the ritual of the examination and to omit entirely the proboscis, body-cavity, and salivary glands. It is also unnecessary, as a rule, to separate the intestine and rectum in the dissection. Consequently, our later examinations were reduced to (1) the excluded fæces, if any, on the slide on which the flea was decapitated; (2) the stomach, on a second slide; and (3) the rectum and intestine, on a third.

It was no part of our task to make a special and detailed study of the

anatomy of the flea, but a few points observed by us incidentally in our dissections may be noted briefly here.

The nervous system, of which some beautiful dissections were made in this laboratory by Major Christophers, I.M.S., consists, as in insects generally, of (1) the brain or supra-œsophageal ganglion-complex, sending off the peri-œsophageal connectives which pass on either side of the œsophagus to connect with the foremost of (2) the three large thoracic ganglia, joined by connectives to form a series which passes on into (3) the abdominal chain of ganglia. It is a very difficult operation to dissect out the brain and the first two thoracic ganglia, but it happens very frequently that in the ordinary dissections of the flea the third thoracic ganglion and the abdominal chain of ganglia are exposed entire and in continuity. It is then seen that the abdominal chain consists of a series of small ganglia terminated posteriorly by a larger ganglion; and further that in the male there are seven smaller ganglia, in the female only six, in the abdominal chain. The larger hindmost ganglion, from which nerves are sent off to the genitalia and rectum, evidently represents a fusion of several ganglia equivalent to the more anterior smaller ganglia. Consequently it is seen that the concentration and fusion of ganglia at the hinder extremity of the ventral chain has proceeded a step further in the female than in the male.

The genitalia consist, in the male, of a conspicuous pair of testes, situated dorsally in the abdomen, and a pair of filamentous glands (prostates?) not unlike Malpighian tubules at first sight, but of slightly smaller calibre, and differing entirely in histological structure. There is no separate seminal vesicle, but each testis is a tightly convoluted tubule, the lower end of which is dilated to contain the ripe spermatozoa. Ducts from the testes and prostates unite to form a median Ductus ejaculatorius, which opens into a large penis of very complicated structure. In the female the two ovaries occupy practically the same position as the testes, but take up much more space and extend forwards to the most anterior limits of the abdomen. Each ovary consists usually of four egg-tubes or ovarioles, but in one specimen there are five ovarioles on each side. The ducts of the ovarioles unite to form the paired oviducts, and these unite in their turn to form the common oviduct. Ventral to the common oviduct lies the unpaired receptaculum seminis, consisting of a brown, chitinous capsule of a peculiar shape. The main body of the capsule is spherical, but gives off a curved, horn-shaped diverticulum, ending blindly. The horn-shaped portion has its concave curve turned towards, and connected by striped muscles with, the spherical part of the capsule. A slender duct of great length, and much convoluted near its origin, arises from the spherical part of the capsule, and runs back to open probably into the distal extremity of the oviduct or into the

genital vestibule. The spherical part of the capsule and duct of the receptaculum are surrounded with unicellular glands, thickly clustered round the capsule and the convoluted portion of the duct, but thinning out and becoming smaller towards the distal end of the duct. The receptaculum, dissected out, stained and mounted for the microscope, is a singularly beautiful object. It usually contains a dense mass of spermatozoa.

The heart is frequently seen in dissections at the hinder end of the body as a delicate filament, which by its own contractions twists and lashes itself about. Under the microscope it appears a delicate tube, beset towards the hinder end by the pericardial cells which are attached to it on either side, right and left, and are crowded together towards the hinder end, but occur more sparingly towards the middle region and are wanting in the anterior third of the heart. The ostia appear to be confined to the posterior region of the heart, but we have not made out their exact number or arrangement. For the pericardial cells, see Minchin (1910).

(b) Notes on the Parasites of the Fleas.

In a former publication one of us (E. A. M., 1910) has described some parasites found in our stock of fleas. The most important was a form to which the name *Malpighiella refringens* was given, occurring, as the generic name implies, in the Malpighian tubules of the flea. Since that time this infection seems to have died out entirely in our fleas, and we have not seen any *Malpighiella* in the fleas dissected by us for the last three years or more. Why this parasite should have died out in our fleas it is impossible to say, but it may be remarked that no conditions could possibly be more favourable for contaminative infection from flea to flea (whether from adult to adult, or larva to larva, or adult to larva, or vice versa) than those in our breeding cages, where vast numbers of fleas in all stages of development are herded together in a confined space. Consequently the disappearance of *Malpighiella* in our cages rather indicates that the fleas do not acquire infection with this parasite by the contaminative method.

In the publication referred to, numerous yeast-like bodies were described and figured from the digestive tract of the flea. Since then we have found organisms of this kind abundantly in smears of the salivary glands (text-fig. 24, p. 642).

In the larvæ of fleas that we have dissected and examined from our cages we have found the gregarine *Agrippina bona* (Strickland, 1912).

The cysticeroids of tapeworms are found not infrequently in the fleas. Nicoll and Minchin (1911) described two species of cysticeroids

from our fleas, representing *Hymenolepis diminuta* and another species of the same genus. We have found the same two species frequently, and also have in our possession specimens of a third species not identified. The cysticeroids appear sporadically, and are sometimes quite common for a period, and then are not found again for a long time. This uncertainty in their occurrence is quite intelligible, since their appearance must be caused by the introduction into the cage of a rat infected with tape-worms, which doubtless infects a large number of the larvæ that later become adult fleas.

The point upon which we wish to lay special stress is the absence in our stock of fleas of any flagellate parasites, and more especially of the leptomonad described by Swingle (1911) under the name *Herpetomonas (Leptomonas) pattoni*. We have been at great pains to convince ourselves upon this point. In the first place we dissected at various times about eighty¹ fleas from the non-infected breeding-cage without finding any flagellates of any kind in them, while flagellate parasites occur in a very large percentage of those known to have fed upon infected rats, though not in all, since the trypanosome often fails to establish itself in the flea, and even when the insect has been fed on a rat with trypanosomes swarming in the blood, they often disappear completely from the digestive tract of the flea within twenty-four hours of its having fed.

We give here three tables (A, B (1), and B (2)) showing the results of dissections of fleas from our stock which had been put upon infected rats, and so had had the chance of acquiring an infection of *T. lewisi*. Fleas do not always feed, however, when given the opportunity to do so, especially in cold weather,² and if the fleas are dissected and examined within twenty-four hours after having been put on the rat (the fleas in all cases having been kept hungry for

¹ As a matter of fact we dissected far more than this, all with negative results, but we have not kept exact records of more than seventy-nine.

² Note especially the twenty-one fleas of November 11th, 1911, in Table A, of which eighteen did not feed. This was due to a sudden cold snap, the first breath, so to speak, of winter.

two or three days previously to being put on), it is quite easy to distinguish those which have been fed from those which have not availed themselves of the opportunity of doing so. In this way useful controls are obtained for determining whether the fleas contained any flagellate infection before being used for putting on the infected rat.

TABLE A.—Fleas Examined within Twenty-four Hours after being put on an Infected Rat, to show the Numbers that had or had not Fed, and the Numbers of those that had Fed but in which no Flagellates were Found.

Date on which the fleas were examined.	Time since fleas put on infected rat.	Number of fleas put on the rat.	Number of fleas apparently not fed and containing no flagellates.	Number of fleas which appeared to have fed and which contained:	
				(a) Stages of T. lewisi.	(b) No flagellates.
5: vii: '10	6 hours	3	1	2	0
19: vi: '11	12 "	10	2	8	0
13: ii: '13	15 "	14	3	11	0
23: v: '11	18 "	4	1	3	0
26: v: '11	18 "	12	1	7	4
25: i: '13	18 "	9	3	5	1
30: i: '13	18 "	5	1	4	0
6: ii: '13	18 "	8	3	5	0
4: iii: '13	20 "	14	12	2	0
10: vi: '10	24 "	4	2	2	0
6: vii: '10	24 "	3	2	1	0
17: iii: '11	24 "	5	1	3	1
15: vi: '11	24 "	11	6	4	1
26: ix: '11	24 "	17	7	8	2
30: ix: '11	24 "	22	2	18	2
3: x: '11	24 "	13	1	12	0
6: x: '11	24 "	14	2	11	1
7: xi: '11	24 "	17	2	11	4
11: xi: '11	24 "	21	18	2	1
17: xi: '11	24 "	17	5	10	2
24: xi: '11	24 "	20	2	12	6
5: xii: '11	24 "	12	3	7	2
12: xii: '11	36 "	6	1	5	0
2: vii: '12	24 "	14	8	6	0
20: vi: '11	24 "	14	3	8	3
Total . . .		289	92	167	30
Percentage . . .		100	31.83	57.79	10.38

From Table A it is seen that of 289 fleas which were put on infected rats, 92 (31·83 per cent.) had not fed and contained no flagellates, 167 (57·79 per cent.) had fed and contained *T. lewisi*, and 30 (10·38 per cent.) had fed but contained no flagellates.

In addition to these negative data we have had the opportunity of comparing our stock of fleas with another stock which was actually infected with *Leptomonas pattoni*. When one of us (E.A.M.) was in Paris in January, 1913, he was very kindly presented by Dr. E. Chatton, of the Pasteur Institute, with some living fleas (*Ceratophyllus fasciatus*) from a stock infected with *Leptomonas pattoni*. These fleas were brought back to London and a fresh breeding-cage colonized with them. The fleas were left to breed for a year, during which time the rat in the breeding-cage was changed frequently, but none of the rats put in acquired any trypanosome-infection. When the fleas were examined at the beginning of 1914, they had multiplied enormously, and were found to contain *Leptomonas*-infections. We did not keep any exact records of our dissections of the *Leptomonas*-fleas, but, roughly speaking, about 50 per cent. of the fleas contained teeming infections. The leptomonads appear to establish themselves in the fleas as readily as does *T. lewisi*, perhaps more so, since, as will be seen from Table B (1), barely more than 14 per cent. of our stock of fleas contained swarming infections when exposed permanently to infection with *T. lewisi* in the infected breeding-cage, and Table B (2), if we count only those known to have fed on an infected rat, not less than six, not more than fourteen days previously, gives but a slightly higher percentage (21·19).

We may conclude, therefore, from a comparison of our stock of fleas with those bred from Dr. Chatton's stock infected with the leptomonad, that, had our stock also been infected with leptomonads, we should not have failed to find fleas containing leptomonads in those fed on clean rats in the first place, and secondly, that the percentage of fleas

TABLE B.—Summary of the Condition of Fleas known to have Fed on Infected Rats. (1) Fleas taken at Random from the Infected Breeding Cage.

Date. (Fleas dissected.)	Trypanosomes.				Experimental infectivity of the flea.
	Fleas. None.		Scanty.	Swarming.	
18:ii:'10	3	1	1	1 (r)	Infection produced by 5 fleas of which 3 were dissected (Table J).
23:iii:'10	4	1	2	1 (s)	
30:iii:'10	6	1	3	2 (s)	
5:iv:'10	6	0	3	3 (1r, 2s)	
8:iv:'10	2	0	1 (si)	1 (si)	
10:iv:'10	2	1	1	0	
22:iv:'10	4	3	0	1 (s)	
3:v:'10	4	4	0	0	
25:vii:'10	5	2	3	0	
27:vii:'10	3	1	2	0	
5:viii:'10	5	2	3	0	One positive (Table K).
6:ix:'10	3	3	0	0	
7:ix:'10	4	3	1	0	
14:ix:'10	3	0	2	1 (s)	
15:ix:'10	3	0	3	0	
22:ix:'10	3	1	1	1 (s)	
23:ix:'10	1	1	0	0	
24:ix:'10	1	1	0	0	
26:ix:'10	2	1	0	1 (r)	
27:ix:'10	3	3	0	0	
28:ix:'10	3	3	0	0	One positive (Table K). Negative (Table K).
29:ix:'10	3	2	0	1 (ir)	
3:x:'10	2	2	0	0	
6:x:'10	2	0	1	1 (r)	
10:x:'10	4	1	2	1 (r)	
13:x:'10	3	3	0	0	
14:x:'10	3*	3	0	0	
17:x:'10	3	1	1	1 (r)	
18:x:'10	3	2	1	0	
24:x:'10	3	3	0	0	
25:x:'10	3*	3	0	0	One positive (Table K). One positive (Table K). All negative (Table K). " (Table K). " (Table K). One positive (Table K). " (Table K). All negative (Table K). One positive (Table K). All negative (Table K). Both negative (Table K). " (Table K).
4:xi:'10	3	2	1	0	
8:xi:'10	3	2	1	0	
15:xi:'10	3	2	0	1 (r)	
16:xi:'10	3	1	1	1 (r)	
21:xi:'10	2	2	0	0	
22:xi:'10	2	2	0	0	

Date. (Fleas dissected.)	Trypanosomes.				Experimental infectivity of the flea.
	Fleas. None.		Scanty.	Swarming.	
29:xi:'10	1	1	0	0	Injection of sal. gl. negative.
30:xi:'10	2	1	0	1 (r)	
1:xii:'10	2	0	1	1 (s)	
5:xii:'10	5	1	4	0	
6:xii:'10	2	0	2	0	
7:xii:'10	2	0	2	0	Both negative (Table K). All negative (Table K).
9:xii:'10	2	2	0	0	
13:xii:'10	3	2	1	0	Fleas infected rat 239, stom- achs rat 257 (Table I).
14:xii:'10	3	2	1	0	
15:xii:'10	6	5	1 (sr)	0	Fleas positive (Table I). Stomachs injected, positive (Table I).
12:i:'11	5*	5	0	0	Negative (Table I).
13:i:'11	6	4	2 (r)	0	Fleas infected rat 251, stomachs rat 261 (Table I).
26:i:'11	6	5	1 (ir)	0	Fleas positive (Table I).
26:i:'11	6*	6	0	0	Stomachs injected, 2 posi- tive.
27:i:'11	5*	5	0	0	Stomachs positive, recta negative.
14:ii:'11	10	2	6	2	
28:ii:'11	10	7	2	1 (r)	
7:iii:'13	8	8	0	0	
13:iii:'13	4	0	1 (sr)	3 (2s, 1sr)	
14:iii:'13	3	0	0	3 (1s, 2sr)	
17:iii:'13	8	8	0	0	
28:iii:'13	9	5	4 (3s, 1sr)	0	
31:iii:'13	7	7	0	0	
4:iv:'13	5	4	0	1 (sr)	
7:iv:'13	6	0	3 (sr)	3 (s)	
10:iv:'13	6	1	4 (1s, 3sr)	1 (sr)	
14:iv:'13	2	0	1 (s)	1 (i)	
Total	249	144	70	35	
Percentage	100	57.83	28.11	14.06	

The batches marked * were fleas of which the stomachs and other organs were kept for injection into rats, and were therefore examined hastily and imperfectly.

s = stomach; r = rectum; i = intestine.

TABLE B.—Summary of the Condition of Fleas known to have fed on Infected Rats. (2) Fleas fed at Definite Periods.

Age of infection in flea (approx- imately).	Date.	No. of fleas.	Fleas containing Trypanosomes.		
			None.	Scanty.	Swarming.
6 hours	14: vi: '10	4	0	1 (s, r)	3 (2s, 1sr)
" "	5: vii: '10	2	0	1 (s)	1 (s)
12 "	28: iii: '11	5	0	2 (s)	3 (1s, 2sr)
" "	19: vi: '11	9	1	2 (1s, 1r)	6 (5sr, 1r)
18 "	21: iii: '11	9	0	6 (s)	3 (1s, 2sr)
" "	23: v: '11	4	1	1 (s)	2 (sr)
" "	26: v: '11	11	4	3 (2sr, 1r)	4 (1s, 3sr)
" "	25: i: '13	6	1	2 (1sr, 1r)	3 (2s, 1sr)
" "	30: i: '13	4	0	2 (1s, 1sr)	2 (sr)
" "	6: ii: '13	5	0	1 (si)	4 (2s, 2sr)
21 "	4: iii: '13	2	0	0	2 (s)
24 "	10: vi: '10	4	2	2 (1sr, 1r)	0
" "	6: vii: '10	3	2	1 (r)	0
" "	17: iii: '11	5	2	1 (s)	2 (sr)
" "	15: vi: '11	5	1	4 (s)	0
" "	27: vii: '11	28*	10 ²	12	6
" "	1: viii: '11	11*	8 ²	1	2
" "	3: viii: '11	29*	12 ²	13	4
" "	30: ix: '11	10	2	6 (4s, 2sr)	2 (1s, 1sr)
" "	3: x: '11	20	2	10 (6s, 4sr)	8 (6s, 1sr, 1r)
" "	7: xi: '11	13	1	4 (1s, 1sr, 2r)	8 (4s, 4 sr)
" "	11: xi: '11	12	1	9 (7s, 2sr)	2 (2s)
" "	17: xi: '11	13	2	8 (5s, 2sr, 1r)	3 (2s, 1r)
" "	24: xi: '11	3	1	1 (s)	1 (s)
" "	5: xii: '11	12	2	6 (4s, 2sr)	4 (3s, 1sr)
" "	9: xii: '11	18	6	12 (s)	0
" "	25: vi: '12	10	0	4 (2s, 2sr)	6 (1s, 5sr)
" "	2: vii: '12	9	2	2 (1s, 1sr)	5 (1s, 4sr)
" "	24: vi: '13	12	0	7 (2s, 3sr, 2r)	5 (sr)
36 "	20: vi: '11	6	1	0	5 (4s, 1sr)
48 "	11: vi: '10	6	5	1 (r)	0
" "	16: vi: '10	5	5	0	0
" "	7: vii: '10	5	5	0	0
" "	18: iii: '11	3	2	0	1 (s)
" "	26: iv: '11	6	2	4 (1s, 2sr, 1r)	0
" "	26: vi: '12	10	1	3 (sr)	6 (5sr, 1s)
" "	3: vii: '12	10	4	3 (r)	3 (1s, 2r)
" "	30: iii: '11	4	3	1 (sr)	0
60 "	11: vii: '12	16	2	12 (1s, 3sr, 8r)	2 (1s, 1sr)
3 days	7: ix: '09	4	2	2 (1s, 1r)	0
" "	16: ix: '09	5	4	1 (s)	0
" "	23: ix: '09	3	2	0	1 (s)

Age of infection in flea (approx- imately).	Date.	No. of fleas.	Fleas containing Trypanosomes.		
			None.	Scanty.	Swarming.
3 days	27: xi: '09	5	1	2 (sr)	2 (1s, 1r)
" "	17: vi: '10	5	5	0	0
" "	8: vii: '10	4	4	0	0
" "	27: iv: '11	10	6	4 (2s, 1sr, 1r)	0
" "	27: vi: '12	10	1	5 (1s, 2sr, 2r)	4 (1s, 3sr)
" "	4: vii: '12	12	6	5 (1s, 1sr, 3r)	1 (r)
3½ "	12: vii: '12	16	4	8 (1s, 7r)	4 (1s, 3r)
4 "	8: x: '09	6	1	4 (sr)	1 (sr)
" "	15: x: '09	8	4	2 (sr)	2 (sr)
" "	19: ix: '10	3	0	1 (sr)	2 (1s, 1sr)
" "	28: iv: '11	10	5	5 (1s, 4sr)	0
" "	5: vii: '12	12	9	3 (sr)	0
" "	18: vii: '12	12	8	3 (r)	1 (r)
5 "	9: x: '09	2	2	0	0
" "	22: vi: '10	2	0	0	2 (sr)
" "	20: ix: '10	3	2	1 (i)	0
" "	29: iv: '11	10	6	1 (s)	3 (r)
6 "	20: vii: '12	12	7	5 (r)	0
7 "	1: v: '11	4	3	1 (s)	0
7½ "	26: v: '13	6	2	2 (r)	2 (1sr, 1r)
8 "	12: x: '09	2	2	0	0
" "	19: x: '09	5	3	1 (r)	1 (r)
" "	17: iii: '13	4	0	1 (sr)	3 (1sr, 2r)
" "	5: v: '13	10	3	2 (1s, 1r)	5 (2sr, 3r)
" "	19: v: '13	6	5	1 (sr)	0
" "	2: vi: '13	6	3	0	3 (r)
" "	9: vi: '13	7	1	3 (1sr, 2r)	3 (sr)
8½ "	27: v: '13	6	2	2 (1sr, 1r)	2 (sr)
9 "	11: iii: '13	6	3	1 (s)	2 (sr)
" "	18: iii: '13	3	1	1 (sr)	1 (s)
" "	13: v: '13	6	6	0	0
" "	20: v: '13	6	6	0	0
" "	3: vi: '13	6	2	3 (1s, 2r)	1 (sr)
10 "	12: iii: '13	4	2	2 (r)	0
" "	19: iii: '13	6	3	2 (1s, 1r)	1 (r)
" "	14: v: '13	8	7	0	1 (r)
11 "	13: iii: '13	2	1	1 (r)	0
12 "	29: vi: '10	1	1	0	0
14 "	1: vii: '10	2	2	0	0
Grand total . . .		609	230	223	156
Percentage . . .		100	37.77	36.62	25.61
Total of six days and over . . .		118	65	28	25
Percentage . . .		100	55.08	23.73	21.19

* Stomachs only examined.

containing flagellates would have been far higher than is shown by our tables, in fleas exposed to infection by *T. lewisi*.¹

(c) Notes on the Histological Structure of the Stomach of the Flea.

We shall have occasion, when describing the developmental cycle of the trypanosome in Part II below, to relate how the trypanosome penetrates into the epithelial cells of the stomach of the flea and goes through a process of multiplication within them. It is a necessary preliminary, therefore, to understanding the effects of the parasites that we should preface our description of their development by some remarks upon the structure and contents of the flea's stomach; and in the following section we give an account of our observations upon these matters, without claiming to have added anything to the scientific knowledge of insect histology.

The histology of the digestive tract of insects has been the subject of

¹ Nöller (1912), discussing the question of the leptomonas-infection of the fleas, remarks, p. 398, that since the larva of the flea acquires the infection, adult fleas bred in a cage can be infected, and that consequently "the arrangement of the experiments ('Versuchsanordnung') of Minchin and Thomson, who used fleas bred in a rat-cage, does not correspond to the requirements ('Anforderungen')." We are at a loss to understand to what this criticism applies or what are the "Anforderungen" to which Nöller refers. At the time Nöller wrote we had published only our three preliminary reports. The first two of these (1910, 1911, 1) refer only to the transmission of *T. lewisi* by fleas, and it is sufficiently obvious that the presence of leptomonads in the fleas could not affect in any way the value or significance of positive results obtained in experiments on the transmission of the trypanosomes, since, *ex hypothesi*, the trypanosome and the leptomonad parasite are in no way connected. Our third report (1911, 2) gave an account of the intracellular multiplication of *T. lewisi* in the flea's stomach, a discovery which Nöller himself has confirmed, and which also would be quite unaffected by the presence of leptomonads in the fleas. Nöller's criticism appears to us, therefore, both premature and superfluous; premature, because our stock of fleas was not, as a matter of fact, infected with leptomonads; and superfluous, because, even if the fleas had been infected with leptomonads, it would have made no difference to the experiments and observations which Nöller criticises.

numerous memoirs, and its general characteristics are very well known. It would be beyond the scope of this memoir to attempt to discuss this subject in detail or to cite the very copious literature dealing with it; but of recent works we may refer more especially to the very excellent monograph of Léger and Duboseq (1902), who have studied the intestinal epithelium of Tracheata from the same point of view as ourselves, that is to say, with the object of describing the changes produced in the epithelium by parasites (gregarines) attacking the cells. None of the insects studied by Léger and Duboseq, however, were of blood-sucking habit and the stomach-epithelium of the flea differs in a number of points from any of the epithelia described by the French authors.

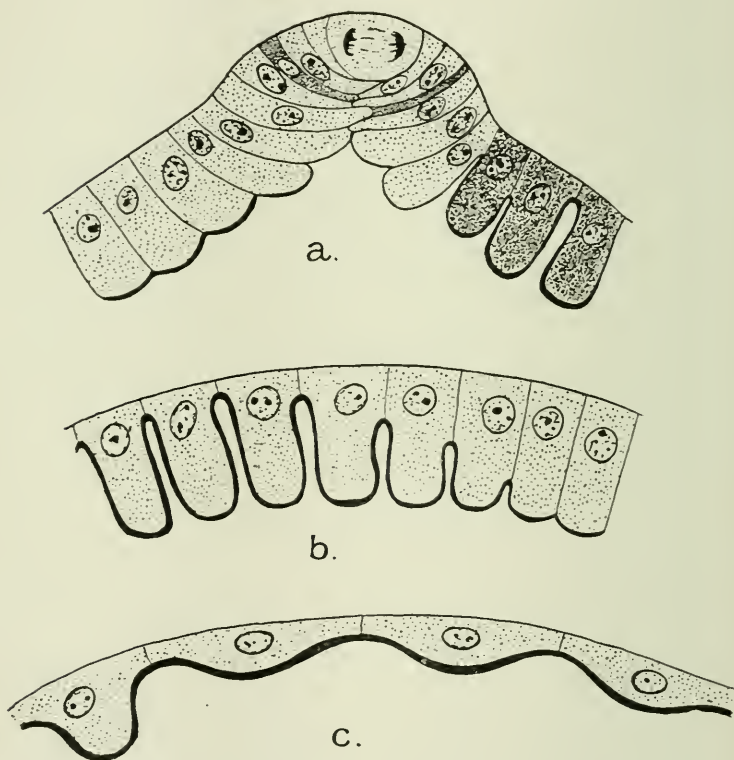
The wall of the stomach consists of the following principal layers, counted from within outwards (Pl. 39, fig. 126): (1) the lining epithelium, (2) a layer of circular muscle fibres, and (3) a layer of longitudinal muscle fibres. In addition to these layers, which are very easily seen, there are to be found also, though by no means in every section, flattened epithelial cells external to, and in contact with, the lining epithelium, between it and the circular muscle-layer: they occur sparingly and far apart, but appear to represent an integral and perhaps primitive constituent of the wall of the mid-gut. Similar flattened cells are found here and there on the Malpighian tubules, the wall of which is similar in histological composition to the stomach-wall if the latter be imagined as reduced to the lining epithelium and the flattened cells alone, without the muscle-layers.

We are concerned here only with the lining epithelium of the stomach, but it may be mentioned in passing that the circular muscle-fibres occur as bands or separate rings with considerable intervals between them, and consequently do not appear in every transverse section of the stomach. The longitudinal muscles are also separated from one another by intervals, a fact at once apparent in the transverse section, in which the muscles are seen cut across and in which it can further be seen in a well-preserved section, that each longitudinal muscle-fibre is connected to its neighbours by a delicate membrane, appearing as a fine line running between each adjacent pair of muscle-bands and forming a delicate sheath or investment round the whole stomach. The circular muscle-layer is continued on into the intestine, where it forms a continuous investment without intervals between the bands; the longitudinal muscles end at the pylorus posteriorly and start anteriorly behind the proventriculus, which has its own system of musculature, running for the most part in oblique bands arranged symmetrically right and left. Each muscle-band in the stomach-wall is a single, transversely-striated fibre, in which an occasional elongated nucleus is seen, embedded in a small quantity of protoplasm.

The following account of the epithelium and contents of the stomach

applies, unless otherwise stated, to sections of the stomach fixed with Flemming's fluid and stained with iron-haematoxylin followed by

TEXT-FIG. 2.



Diagrammatic representations of sections of the epithelium of the flea's stomach, to show the various conditions: *a*, to show a section through an epithelial crypt from which clear, regenerated epithelium is arising on all sides, while on one side three black, degenerated cells are seen (compare fig. 316, Pl. 44); *b*, to show the manner in which the border of the cells arises in relation to the gradually-developed separation of the cells from one another; *c*, to show the transition from the ordinary, columnar type of epithelium to the flattened type.

Lichtgrün-pieric (see the following section dealing with technique). If such a section through a number of stomachs—all taken from a batch

of fleas dissected at the same sitting and at the same interval of time after having fed on the infected rat, all preserved in the same way, all stuck on the same slice of liver, cut and stained simultaneously—be examined even in the most cursory manner, very considerable differences are seen between the stomachs in one and the same microtome-section. These differences affect both the epithelium and the contents. The epithelium varies, in the first place, in the form of the cells, from flattened to columnar, and secondly, in the staining reactions of the cells. The contents of the stomach, that is to say the blood-débris, vary greatly in colour, staining in some cases deep opaque black, or less deeply in various shades of grey, in other cases, however, bright yellow.

The variations in the form of the epithelial cells are to be ascribed to the differences in the degree to which the stomach is dilated by the ingested blood. In a gorged flea the distension of the stomach stretches the epithelium until the cells become thin and flattened; but when the flea is hungry, or has taken in a small quantity of blood, or when the quantity ingested has become reduced by digestion and absorption, the epithelium resumes what may be considered its normal columnar form. Every gradation between the flattened and columnar conditions can be found in different sections or in different parts of one and the same section.

The variations in the staining reactions of the epithelial cells depend, in the first place, on the age or senescence of the cells. It is a matter of common knowledge that the lining epithelium of the mid-gut of insects is continually being thrown off and regenerated. The ordinary epithelial cells do not multiply and no mitoses are ever found in them; the centres of regeneration are the so-called epithelial crypts, each representing morphologically a small diverticulum of the epithelium in which the approximation of the cells usually obliterates the cavity and produces a solid, bud-like mass of cells (Text-fig. 2, *a* and Pl. 44, figs. 314, 316). When a flea's stomach, containing a certain amount of ingested blood, is plunged into a fixative, the epithelial crypts are very easily seen with a hand-lens or with the naked eye as little opaque white spots in the semi-transparent stomach-wall, very conspicuous against the reddish-brown background of the stomach-contents. In the sections it is common to find mitoses in such a crypt, especially towards its fundus (Text-fig. 2, *a*). As the cells multiply they are pushed upwards to the general level of the epithelium and outwards from the crypt to replace the old epithelial cells which, having degenerated, are cast off from the wall into the lumen of the stomach, and are digested there.

The young, freshly-regenerated epithelial cells have the cytoplasm clear, staining light-grey, and are relatively poor in granulations: the older cells, on the contrary, have the cytoplasm full of granules that stain very deeply, until finally the whole cell, including its nucleus

becomes black and opaque. Consequently, the epithelia of different stomachs show very varied appearances. A recently regenerated stomach will show clear epithelium all round, and, according to the time that has elapsed since regeneration, there may be no detached cells in the lumen of the stomach, or there may be a certain number of detached black cells, or there may be still, here and there, isolated black cells or patches of such cells *in situ* in the epithelium or in process of being cast off from it. On the other hand, a stomach which is about to be regenerated shows very dark epithelium all round, and in places this may be in process of rejection and replacement from the crypts, in which the cells have clear cytoplasm. The condition of the epithelium may vary in different parts of the same stomach, and from what we have observed we have gained the impression that the regeneration proceeds usually from before backwards, so that the anterior part of the stomach is further advanced in degeneration or regeneration, as the case may be, than the posterior region. We find in our preparations all possible conditions of the epithelium in different stomachs in one and the same microtome-section, and we have not been able to establish any definite relation between the feeds of the flea and the regeneration of the epithelium, but we have not paid sufficient attention to this point to be able to state positively that no such relation exists; the differences seen in the epithelia of flea-stomachs examined at the same interval of time after feeding may be due to inequalities in the rate at which digestion proceeds.

In addition to what appears to be the normal process of senile decay, in which the cells take up the iron-haematoxylin stain very deeply and become black and opaque, we have observed a second mode of degeneration, which we are inclined to ascribe to the action of the trypanosomes, since in all cases where it occurs in our preparations there are trypanosomes to be found in the stomach, and frequently in the degenerated cells themselves. In this second type of degeneration the black-staining granules in the cell diminish in quantity, without, however, disappearing entirely, while the cytoplasm of the cell stains yellow (Pl. 39, fig. 133; Pl. 40, fig. 140); hence we have generally referred to this condition in our notes as "yellow necrosis." In all the stomachs in which we have found it the blood-débris is also stained yellow, and it is often very difficult to make out the precise boundary of the necrosed cell-body, or to distinguish the cells from the débris when they lie free in it (Pl. 39, fig. 125), except by the presence of the nucleus and of a certain number of black granules in the cytoplasm of the necrosed cell. Indeed, our first impression was that the yellow colouring matter of the blood had in some way penetrated into the cell and stained its cytoplasm, but there can be no doubt that this idea is an illusion and that the yellow colour, both of the blood-débris and of the necrosed cells, is due to the picric

acid in the Lichtgrün-picric staining combination, though it is, of course, possible that the substance, whatever it may be, which stains yellow in the blood-débris may have become infiltrated into the dead cells and given them their peculiar staining properties.

The variations in the staining reactions of the contents of the stomach are more difficult to explain. There appear to be two types of staining after the use of the iron-hæmatoxylin-Lichtgrün-picric combination, one in which the contents stain in various shades of grey up to black, with a greenish tinge, and one in which they stain a bright lemon-yellow. It is not possible to bring these two types of staining into one series, as there is no transition between them; the grey-black and the yellow types occur side by side in different stomachs in one and the same microtome-section, and each stomach shows either the one or the other condition through the whole series of sections. So far as our observations permit us to generalise, the grey-black series represent the normal stages of the digestion of the blood; the yellow reaction appears to be due to some abnormal condition.

The blood ingested by the flea is very soon affected by the digestive action of the stomach, and the red corpuscles cease to be recognisable within a few hours after digestion. In the middle period of digestion, that is twenty-four hours, or thereabouts, after feeding, the blood has become thick, viscous and brick-red in colour, and contains immense numbers of irregularly shaped grains of all sizes, but for the most part coarse and large. Towards the end of digestion, forty-eight hours or so after feeding, the stomach contents are fluid and watery, dark brownish-black in colour, and the grains are much diminished in number and in size.

In the sections, taking first the grey-black series, the blood in the earlier phases of digestion (eighteen to twenty-four hours) usually consists of densely-packed grains and spherules, varying in size from very coarse to very fine, and staining intensely black. Between the grains there is visible a coagulated albuminous matrix, stained greenish with the Lichtgrün-picric combination. The stomach-contents fill the whole section and adhere closely to the epithelial cells, penetrating down between them when they have the columnar form, but in the centre of the section there is generally a clear patch, circular in outline, which is seen to owe its clear appearance to the fact that coarse grains are absent, and it consists only of the alluminous matrix with finer granules. Hence the digestion, or more probably the passage backwards towards the rectum of the indigestible remnants, of the blood-débris appears to proceed from the centre of the section—that is to say, from the axial region of the stomach—towards the periphery.

As the digestion proceeds, the grains in the débris become smaller

and stain less deeply; consequently the stomach-contents stain grey, in varying shades of darkness, while the matrix still shows the greenish hue. In stomachs thirty-six hours after feeding the contents of the stomach are generally greatly diminished in quantity, and are absorbed in the centre of the lumen, leaving a clear space of variable form, while round the periphery the greenish-grey débris adheres close to the epithelium. Leucocytes, especially the polymorphonuclear forms, can be recognised in the blood twenty-four hours after feeding, but at thirty-six hours we have not found them. Owing to the lighter tints of the stomach-contents at thirty-six hours, the trypanosomes free in the blood-débris can be seen more easily, in contrast with the earlier state of affairs.

In the yellow stomachs the contents appear at first almost uniform, but on close examination they are seen to consist of closely-packed granular substance, all of which, both granules and matrix, is coloured by the picric acid in the staining combination used. The first point that strikes one immediately is that the contents in such stomachs are large in quantity and fill the whole stomach, or show but a slight amount of absorption towards the centre of the section, even at thirty-six hours, when the contents of the grey-black stomachs are considerably diminished. The epithelium of the yellow stomachs may vary from the flattened to the columnar form, but the normal cells stain grey or black, in sharp contrast with the yellow contents.

It seems obvious from these data that the yellow stomachs represent an abnormal condition; we have endeavoured, not very successfully, to find a relation between this condition and either the presence of trypanosomes, on the one hand, or the state of the epithelium on the other.

In the yellow-staining stomachs which we have studied we have found trypanosomes to be present in the stomach in every case except one, and in that case there were attached clumps of crithidial forms immediately behind the pylorus, showing that the stomach-phase was over. But on the other hand, we have found the grey-black condition of the contents in well-infected stomachs also, showing at least that, if the yellow condition is in any way due to the parasites, they do not always produce that effect. On the other hand, in those cases in which we have found no trypanosomes at all, either in the stomach or outside it, the contents are always in the grey-black condition. A significant circumstance is, perhaps, the fact that we have only found the "yellow necrosis" of the cells in stomachs with yellow-stained contents.

As regards the condition of the epithelium, we have found the yellow condition of the contents associated (1) with epithelium black all round and in process of being cast off, or (2) with epithelium mostly clear, but with black patches of cells in situ or detached; in one such stomach the

first condition is found in the anterior half, the second in the posterior. We can state, therefore, that in our experience the yellow-stained contents occur only in stomachs about to be regenerated, or in process of regeneration, or very recently regenerated. But, again, we have found the grey-black condition in stomachs that appeared also to have undergone regeneration very recently, which makes it difficult to correlate this condition with the process of regeneration. The question of the significance and cause of the yellow-staining condition of the stomach-contents must be left an open question at present; the data to hand do not suffice for drawing decisive conclusions, and it would lead us too far to attempt further investigations upon this problem. On the whole, however, it seems at least probable that we are dealing with an abnormal state of the digestive processes towards which the trypanosomes are a contributory cause, if not the sole one.

As already stated, the different conditions of the stomach-contents described above are those seen after staining with iron-hæmatoxylin and Lichtgrün-picric. After the use of Giemsa's stain the colour of the contents differs considerably in different cases.

Most of our sections stained with Giemsa were fixed with Maier. In those in which the trypanosomes were best stained and show the flagella clearly and sharply the grains and spherules of the débris are coloured for the most part orange-pink, especially in those stomachs in which the digestion of the blood is further advanced (figs. 109, 113, Pl. 38); in the earlier stages of digestion many of the larger grains and masses in the débris are stained deep purple, making the contents of the stomach more opaque. In one of our series preserved in Flemming, consisting in all of seven slides, the first six were stained with the iron-hæmatoxylin-Lichtgrün-picric combination, the seventh with Giemsa's stain; on this seventh slide there are sections of four stomachs, two of which, on the other six slides, show grey-black contents, while the remaining two have the contents yellow in colour. In the Giemsa-stained slide the blood-débris shows a coloration very different after Flemming to that which it shows after Maier, being stained a bluish-green tint. The stomachs of the yellow type are slightly more blue in tint than those of the grey-black series, but otherwise the difference between them is but slightly marked.

Having now described the chief variations in the conditions of the stomachs and their contents, or at least those differences which are obvious upon the most cursory inspection of the sections, it remains to give a more detailed account of the epithelial cell. In any given stomach the cells show great individual variation in form and structure, but, nevertheless, it is not possible to divide them into distinct classes. There are no special glandular or secreting cells, as described by Léger and Duboscq in other insects, and all the cells of the general epithelium

of the stomach of the flea are to be regarded as equipotential, the differences visible between them being merely the expression of varying physiological conditions in relation to their changing environment, on the one hand, or to their constitutional vigour or senescence, on the other. Hence it is possible to give a generalised description of the cells, beginning first with the normal, healthy cell and dealing afterwards with the changes it undergoes in the process of degeneration.

The epithelial cells are produced, as already stated, in the "crypts of regeneration," which have been described in various insects by Léger and Duboscq. In the flea these structures appear usually as solid, bud-like cell-masses that often project outwards from the wall of the stomach to a considerable extent (Pl. 44, figs. 314, 316) beyond the level of the muscle-layers, which pass on either side of them. Internally the crypts do not rise up beyond the general level of the epithelium. The closely-packed cells of the crypts show distinct limits, and do not form a syncytial mass of protoplasm, as described by Léger and Duboscq (l. c., p. 410) in the larva of *Anthrenus verbasci*, for example. At the fundus of the crypt mitoses are often found, sometimes in two cells simultaneously in the same crypt; in other cases all the nuclei are in the resting state. Doubtless the crypts have periods of active multiplication, alternating with periods of repose, as in other insects. The crypts are often seen to be marked off from the general epithelium by slender dark cells, the "cellules de recouvrement" described by Léger and Duboscq (l. c., Pl. II, fig. 2, *c. r.*; p. 388). The crypts appear to have the monopoly of cell-production in the stomach of the flea. We have not found basal cells, "cellules de remplacement," in the general epithelium.

By multiplication and increase in their numbers the cells are pushed outwards on all sides from the crypt to take their place in the general epithelium (Pl. 44, fig. 316, and Text-fig. 2*a*). The young epithelial cells seen in the immediate neighbourhood of the crypts are columnar cells, roughly rectangular in form, and generally about twice as high as they are broad. The lateral boundaries of the cell are approximately parallel, and each cell is in contact with its adjacent neighbours for its whole length. The free, apical surface of the cell is convex, and on this side is developed a very distinct, thick border, at first covering only the upper surface of the cell, which projects like a dome towards the lumen of the stomach.

The further development in the form of the cell consists in an extension of the upper free surface, brought about by the cells becoming free and separated from one another at their sides, first at their apices and then downwards along almost the whole length of the side of the cell, till finally each cell is connected with the adjacent cells only by a narrow isthmus at its base (Text-fig. 2*b*). As the cell

becomes free the border develops also on the exposed surface, so that, instead of being confined to the apex of the cell, it extends down the vertical sides also (Pl. 40, fig. 136). This process of separation between the cells has an obvious significance in connection with the process of flattening which they undergo when the stomach is dilated after feeding; it can be regarded as an adaptation to the blood-sucking habit. When the flea gorges itself each cell is so stretched that its tallest part in the vertical direction is scarcely thicker than the nucleus, which bulges out the middle part of the cell in an even curve towards the lumen of the stomach, while towards the periphery the vertical height of the cell diminishes to the isthmus connecting it with its neighbours (Text-fig. 2*c*). As the cell resumes the columnar form the nucleus remains at or near the base, as a rule, and the cytoplasm of the cell is heaped up over it. In the extreme columnar form the apex of the cell is generally slightly expanded, the middle region more narrowed, so that spaces are left between adjacent cells, into which a considerable quantity of blood-débris penetrates (Pl. 39, fig. 126). The nucleus is usually situated at the base of the cell, but occasionally towards the apex (Pl. 40, fig. 136). The border clothes the whole free surface of the cell, whether flattened or columnar, and is of considerable thickness over the apex and the sides, becoming thinner as it approaches the isthmus, but in the columnar form of the cell, when its apical region is expanded, the border may be thinner, as if stretched, at the apex of the cell (Pl. 40, fig. 144).

The blood-débris has a great tendency to adhere closely to the border, so much so that the border is often more sharply marked off from the cell-contents within than from the blood-débris without, in the sections, but places can be found occasionally where the blood-débris has split away from the epithelium, leaving the border distinct and sharp. The border appears usually homogeneous and refringent, though in some preparations indications are seen of a vertical striation, as if it were composed of little darkly-stained rods, placed at right angles to its two limiting surfaces, and separated by intervening substance of lighter colour (Pl. 39, fig. 129, and Pl. 40, fig. 142). After sublimate-fixation the border is colourless, but when stained with iron-hæmatoxylin the blood-débris adhering to it often hinders the extraction of the stain and at these spots it remains black; when the hæmatoxylin is extracted it tends to take up the green from the Lichtgrün-pieric mixture (Pl. 40, fig. 147). With Giemsa after sublimate-fixation it stains a pinkish-yellow. After Flemming-fixation the border is yellowish, as if tinged by the chromic acid in the mixture, and when this fixation is followed by the Giemsa-stain the border is coloured green (Pl. 38, figs. 99-103). There is no "bordure en brosse," or palisade of stiff rod-like cilia, external to the border, as in many insects. The condition in the flea

more resembles that figured by Léger and Duboscq for *Scelopendra* (l. c., pl. vi).

The border is evidently a fairly tough structure since in teased up stomachs examined fresh, the borders of cells are often seen quite empty, but retaining their shape, like shells.

The nucleus of the epithelial cell calls for no special comment: as can be seen in our figures, it is rounded or oval, with the typical structure seen in tissue-cells, namely, a distinct membrane, a reticulum containing chromatin-grains of various sizes, and one or more nucleoli which stain black, like the chromatin, after iron-hæmatoxylin. Mitoses of the usual type are found commonly in the crypts of regeneration, but we have never seen the slightest evidence of nuclear division in cells forming part of the general epithelium outside the crypts.

The cytoplasm of the epithelial cell varies at different ages. In the youngest cells bordering the crypts the cytoplasm appears more or less homogeneous and finely granular in all parts of the cell; it stains light purplish-grey or grey-black after iron-hæmatoxylin, bluish-purple after Giemsa, and no coarse granulations are to be seen. In the fully developed cell the cytoplasm has undergone local differentiation; round the nucleus, in the basal half of the cell, it has a denser texture, but above the nucleus, in the apical region, it has become of looser consistence, more spongy, so to speak, in appearance, with irregular spaces (Pl. 39, figs. 126, 127, Pl. 40, figs. 136, 144), containing fluid in the living condition, and transversed by strands of protoplasm disposed irregularly. The more the apical part of the cells is expanded the more watery its contents appear. Sometimes the apical region appears almost empty in the sections, with only a few traces of cytoplasm close under the border and at the sides. It is in this region in which the stages of the trypanosomes are most often found, and into which the parasites first penetrate.

In addition to these changes in the cytoplasm, numerous grains and enclosures of various kinds make their appearance in it. A detailed study of these granulations would require a lengthy investigation, an expenditure of time and trouble, that would go beyond the scope and objects of this work. We must confine ourselves to a brief summary of the appearances seen in our sections, without attempting to give physiological explanations of the various conditions seen. It is obvious that the bare observation that a granule is stained black by iron-hæmatoxylin or red by Giemsa's stain does not permit very far-reaching conclusions as to its nature or function in the cell; bodies of most diverse properties might agree to this extent in their reactions.

The first granulations to appear are minute grains which, whatever the fixation, Flemming or sublimate, stain black after iron-hæmatoxylin and red after Giemsa. They are seen at first chiefly at the sides of the

nucleus, between it and the cell-wall and extend up the sides of the cell close under the border. Scanty at first in the apical spongy part of the cell, they are soon deposited in this region also, appearing often in considerable numbers and varying in size from small granules to conspicuous grains, and even large masses (Pl. 40, figs. 136, 137.) The larger grains are seldom homogeneous, but appear as rings, black or red, as the case may be, with clear centre, apparently hollow (Pl. 38, fig. 99). Those of still larger size show, especially after Giemsa, darker and lighter parts disposed in various ways; inside the peripheral deeply-stained shell there may be darker grains or patches. After iron-hæmatoxylin, however, the whole mass may be opaque black, but usually shows lighter inner portions. The extent to which these granulations are developed varies in different stomachs, doubtless in relation to their secretive or absorptive activity at the moment of preservation. When a number of stomachs are cut in the same block, one stomach all through the series may show the cells clear and very free from granulations, while another stomach shows nearly every epithelial cell loaded with coarse grains in its apical region.

The red grains, as they may be termed from their distinctive reaction to Giemsa's stain, appear to be always present in greater or less quantity in the fully-developed cells of every stomach. In addition there are often found, lodged in the apical spongy region of the cell, masses of relatively large size which do not retain the iron-hæmatoxylin stain firmly throughout their substance, and consequently appear for the most part light grey in colour after this stain (Pl. 40, figs. 145, 146); after Giemsa they are either scarcely stained at all, appearing a sort of neutral tint, or they are coloured bluish-purple in various shades, sometimes very deeply, with streaks and blotches more reddish in tint (Pl. 38, fig. 102). These masses vary considerably in size and contour, and show differentiation of their substance into lighter and darker parts. With superficial examination they often simulate the intracellular stages of the trypanosomes to a remarkable degree, especially in the living condition, when they are often very conspicuous; for a long time we confused them with the spheres in the freshly teased-up stomachs, and spent much time watching them in the expectation, never of course fulfilled, of seeing them perform the characteristic movements. After we had made smears of stomachs in which these bodies were abundant, without finding any intracellular stages of the trypanosomes in such preparations, we came to the conclusion that these motionless spheres (as they appeared to be) were merely cell-products, and referred to them in our notes as "pseudospheres." Even in sections the pseudospheres often mimic the true spheres and might be confused with them at first sight, but only by an inexperienced observer who had never seen the actual intracellular stages of the trypanosome in the epithelium. The

idea occurred to us at one time that some of the pseudospheres might possibly be degenerated stages of the trypanosomes, destroyed, and in process of absorption, within the epithelial cells into which they had penetrated: but we have found no decisive evidence for this. It is most probable that the pseudospheres represent secretion-masses formed by the cell itself.

In some of the stomachs, especially in those preserved about twenty-four hours after feeding, there are to be seen dense and very conspicuous accumulations of coarse grains in the epithelial cells immediately below the border (Pl. 38, fig. 98, Pl. 40, fig. 147). The grains in question are especially distinct after fixation with Maier's fluid: they are more difficult to make out in the stomachs fixed with Flemming. The grains resemble very closely those of the blood-débris adherent to the border external to the cell, so much so that the first impression gained is that the débris has been absorbed into the cell through the border. It is easy to imagine this after iron-hæmatoxylin, which stains both these granules and the débris very black after sublimate-fixation (fig. 147); but the Giemsa-stain colours the grains within the cell differently from the débris (fig. 98), and when the digestion of the blood has gone beyond a certain point the grains inside the cell may be stained much darker with iron-hæmatoxylin than the grains in the blood-débris. It is improbable that the coarse grains of the débris would pass bodily through the border, which is to all appearances a dense, tough structure: but it is probable that these grains are formed in the cell in direct relationship with the process of absorption of nutriment from the blood.

Amongst the enclosures of the epithelial cell must be mentioned finally peculiar yellow grains which occur with great frequency in some stomachs, not at all in others. Their presence or absence is in no way connected with that of the trypanosomes, and they occur both in normal as well as in degenerating cells, though perhaps more abundantly in the latter. In the Flemming-iron-hæmatoxylin sections these grains have a brownish-yellow tint, often with a darker shell (Pl. 40, fig. 141). They vary in size from small granules up to the large grains reaching as much as 13μ in diameter (fig. 142). Their tint also varies in depth, being usually much lighter in the larger grains. With Giemsa, after Flemming, they are stained bright green (Pl. 38, fig. 103), probably as the result of a blue stain (azure) imposed upon their original yellow tint.¹ These yellow bodies are very similar to, probably identical

¹ A similar result is seen in the chitinous spines of the proventriculus in sections stained with Giemsa; the cuticle at the base of the spine is stained red, but that of the spine itself, from near the base to the tip, is coloured emerald green. The unstained spine is yellow in tint. Com-

in nature with the enclosures characteristic of the pericardial cells. As one of us has described elsewhere (E.A.M., 1910), the pericardial cells of the flea may be so crammed with yellowish-brown grains and spheres that the cell becomes visible with the naked eye as an opaque black spot through the integument of the living flea. In some of our stomach-sections there are also casual sections of pericardial cells which have been pulled out of the flea together with the stomach, so that we have had the opportunity of making a direct comparison between the yellow grains in the epithelial and pericardial cells. The yellow grains are probably an excretory product, eliminated by the flea under certain physiological but apparently normal conditions, and elaborated either in the epithelial cells, to be cast out into the lumen of the stomach, or in the body-cavity, to be taken up by the pericardial cells.

We come now to the process of cell-degeneration which occurs in the effete, senile epithelial cells. This process is very different in the flea's stomach from that described by Léger and Duboscq in various insects, none of them of blood-sucking habit. It is described by these authors as a "*Dégénérescence mucoïde*," an infiltration of the cells with mucoid substance. In the flea's stomach the process appears to be more of the nature of a fatty degeneration, combined perhaps with a mucoid infiltration.

In our sections fixed with Flemming's fluid and stained with iron-haematoxylin the intensely black, often perfectly opaque, degenerated cells, which are seen frequently detached completely or in process of detachment from the epithelium, are very distinct from the clear, lightly-stained cells originating from the crypts of regeneration and taking the place of the degenerated cells (Pl. 44, fig. 316). In some of our sections the stain has been over-extracted: the trypanosomes have become ghosts, faintly visible only to the practised eye, the nuclei of the epithelial cells are pale, and even the blood-débris has had its usually intense black stain reduced to a shade of brown; but the black grains and masses in the epithelial cells remain as black as ever, showing that they do not owe their colour to the stain but to the fixation, that is to say, to the osmic acid in the Flemming's fluid. Such preparations, spoilt for other purposes, are very useful for showing the gradual process of deposition of the blackened grains. First they appear as fine granules round the nucleus, near the base of the cell (Pl. 40, figs. 143, 144). Next, other, and for the most part larger masses, are deposited in the cytoplasm above the nucleus. The cell then becomes gradually filled up with black grains from below towards the apex; often an

pare also the green stain of the border, mentioned above, after Flemming and Giemsa, evidently due also to the super-position of a blue dye upon a yellow ground.

empty space is seen at the apex, immediately below the border (Pl. 40, fig. 138), but finally this, too, is filled up and the whole cell becomes an opaque black mass (fig. 139).

Very instructive is one of our series preserved in Flemming, in which there is one stomach in which nearly all the epithelium is degenerate. The sections of this stomach are spread over seven slides, six of which were stained with iron-haematoxylin, while the seventh, on which are sections through the hindmost region of the stomach, was stained with Giemsa. On this slide the degeneration is not so far advanced as in the more anterior region of the stomach, and in different parts even of the same section the following conditions are to be found: (1) Cells of normal type, with clear cytoplasm containing a few red granules (Pl. 38, fig. 99); (2) cells with cytoplasm of a darker bluish-purple tint, with many more red granules and amongst them a few coarser grains intensely black in colour (Pl. 38, fig. 100); (3) cells in which both the red and the black grains, but especially the latter, are greatly increased in number, leading up to (4) opaque black cells in which nothing can be focussed clearly. The black grains, it is obvious, can only owe their colour to the action of the osmic acid in the fixation, and must therefore be of a fatty nature. On the other hand there is also a marked increase of the red grains in the degenerating cells, indicating, perhaps, that in addition to deposition of fat, there is also a tendency to mucoid infiltration, as described by Léger and Duboscq. The darker tint of the cytoplasm, in so far as this is not an optical effect due to crowding of the grains, indicates that it becomes impregnated with the substances produced in the process of degeneration.

The deposition of the fat round the nucleus in the first instance indicates that the nucleus takes an active share in the process, and this is borne out by the fact that the nuclei themselves become very dark in the degenerating cells and are sometimes quite opaque.¹

In sections of stomachs fixed with sublimate mixtures the blackening of the degenerating cells seen in the Flemming-fixed sections is conspicuously absent, so that at the first glance it is difficult to pick out the senile portions of the epithelium. More careful study of the sublimate sections shows that here the degenerated epithelium is distinguished from the regenerated by its pale, empty appearance, owing to the fat-grains having entirely disappeared, leaving empty spaces to mark their former position. This is best seen in stomachs fixed in sublimate-acetic, since, after sublimate-alcohol mixtures (Maier's and Schaudinn's) the cells are often much deformed and shrunk. In a favourable spot it is seen that the young cells, freshly produced from

¹ Léger and Duboscq have noted also that the mucoid substance is deposited first in the nucleus.

the crypts, have denser cytoplasm filling the cell throughout, except in the apical expanded portion of the cell; the cytoplasm stains deep grey or neutral tint after iron-hæmatoxylin and shows relatively few enclosures. The senile cells, on the contrary, are full of cavities, so that the cytoplasm has a spongy appearance throughout the cell and not merely in its apical region; and scattered through the spongy cytoplasm are grains, fine or but moderately coarse, which are stained black after iron-hæmatoxylin, red after Giemsa.

The difference between the senile cells after the two methods of fixation is easily explained if the grains deposited in them are principally fat. In all the sections alike the fat has been dissolved away during the process of imbedding in paraffin. In the Flemming-fixed sections, however, each fat-grain has reduced the Os O_4 to metallic osmium, and consequently is represented in the sections by a black mass, a model of the fat-globule in metallic osmium. In the sublimate-fixed sections no such reduction takes place, and the fat-globule is represented by an empty space; only the mucoid grains (if we are right in calling them so) remain in the cytoplasm, stained red or black according to the stain used.

It should be mentioned finally that after sublimate-fixations the blood-débris is stained very much blacker by iron-hæmatoxylin, and holds the stain much more tenaciously than after Flemming-fixation. This is especially true of that part of the débris which penetrates down between adjacent epithelial cells, and which often remains jet-black after all the rest of the débris has become pale in tint. In consequence the cells of the columnar epithelium in sublimate-fixed sections are often seen to be separated by black masses, which careless observation might confuse with the black stain of the degenerated cells after Flemming-fixation, especially when, as often happens in such sections, the main mass of the débris has shrunk away from the epithelium into the centre of the stomach-lumen. Such a mistake could only be made, however, with powers too low to discern that the black masses are between the cells and not in them.

The degenerated cells are thrown off boldly into the lumen of the stomach, which often contains great numbers of them in the blood-débris. There they are doubtless digested and absorbed along with the other contents of the stomach. Léger and Duboscq described a process of mucoid degeneration in which the entire cell, having a remarkable and deceptive resemblance to a gregarine, is engulfed by a basal cell: ultimately the latter also degenerates, and is thrown off with the cell it has taken in (l. c., p. 451). We have seen nothing of this sort in the flea, in which basal cells do not occur in the epithelium of the stomach.

(3) TECHNIQUE.

We have already described above our methods of dissecting the flea and extracting from it the organs which it is required to examine for the presence of stages of *T. lewisi*. Here we propose to describe the methods by which the trypanosomes, when found, were preserved as permanent preparations for microscopic study.

The organs of the flea, extracted in the manner described above, are at once examined carefully under the microscope for the presence of trypanosomes in their various phases of development. When trypanosomes were found in any of the internal organs, after note had been taken, or sketches made, of their forms, position, and other points of interest, we proceeded to make permanent preparations of them. For this purpose the coverslip is carefully raised up, by means of the pair of fine needles that were used in the dissection of the flea, lifted off, and dropped at once with wet surface downwards into a suitable fixative. The slide is then handed to the collaborator or to an assistant, who places it bodily into a tube containing a small quantity of four per cent. solution of osmic acid. In the tube the slide remains about ten to fifteen seconds, tightly corked up, in order to fix the trypanosomes with the vapour of osmic acid. Subsequently the slide is fixed with absolute alcohol for about fifteen minutes and stained with Giemsa's stain in the usual manner.

For the fixation of the coverslip-films we used, in the earlier periods of our investigation, either Schaudinn's fluid (corrosive sublimate, saturated solution in distilled water, 100 c.c.; absolute alcohol, 50 c.c.; glacial acetic, a few drops) or sublimate-acetic (HgCl_2 saturated in H_2O , 95 volumes; glacial acetic, 5 volumes). Both these fixatives gave results about equally good; it is difficult to choose between them. Latterly, however, we used only Maier's modification of Schaudinn's fluid (distilled water, 200 c.c.; absolute alcohol, 100 c.c.; sodium chloride, 1.2 gm.; HgCl_2 , 10 gm.), since this appeared to us to give better preservation, and, in particular,

less shrinkage of the bodies of the trypanosomes, than the others. The fluid being put into a large watch-glass, the coverslip is dropped into it with the film downwards. The coverslip usually sinks in the fluid and then rests on its corners on the rounded bottom of the watch-glass, so that the film itself escapes any friction or injury. The coverslips are left in the fixative from ten minutes to half-an-hour or longer (the exact time appears to be immaterial), and are then passed through 50 and 70 into 90 per cent. alcohol, where they can be kept until it is convenient to stain them.

The coverslip films were stained almost invariably with Heidenhain's iron-hæmatoxylin, using $3\frac{1}{2}$ per cent. iron-alum solution and $\frac{1}{2}$ per cent. hæmatoxylin-solution, both in distilled water. The film, after having been brought down through graded strengths of alcohol (80, 70, . . . 10 per cent.) to water was left about twenty-four hours in the iron-alum, then as long in the hæmatoxylin. Immediately before using the hæmatoxylin-solution a few drops of a saturated watery solution of lithium carbonate was added to it, drop by drop, until the solution, when shaken up, was a bright claret-red colour. After the film had been twenty-four hours in the hæmatoxylin-solution the differentiation of the stain was carried out under control by the microscope in a weak (light brown) watery solution of iron-alum. When differentiation was complete the film was washed in a current of tap-water for at least twenty minutes, then rinsed in distilled water and brought up through graded strengths of alcohol to absolute alcohol. At this stage the coverslip was usually dipped for a moment into Lichtgrün-picric solution (Lichtgrün, 1 grm.; picric acid, $\frac{1}{2}$ grm.; absolute alcohol, 100 c.c.), then washed again in absolute alcohol, passed through xylol, and mounted in pure xylol-balsam on a slide. The Lichtgrün stain must be used very rapidly, as it stains intensely.

In this way two preparations were obtained of the contents of each organ—one on the coverslip, the other on the slide—and as a rule trypanosomes were found more or less

abundantly on both of them, so that it was possible to compare corresponding phases of the development prepared by distinct methods of technique. It is very important, however, that the operation of removing the coverslip and fixing the films should be performed very rapidly and expeditiously, in order to avoid any drying taking place. The coverslip is particularly liable to dry, since the film of liquid that adheres to it is very thin; the slide, on the contrary, does not dry so quickly. A coverslip that has dried before fixation is quite useless for staining by the iron-hæmatoxylin method; the trypanosomes acquire a characteristic shiny appearance, as if they had been glazed, and when the stain is extracted in order to differentiate the preparation, it does not come out of the cytoplasm evenly, but gives a blotchy appearance, with no sharp differentiation of the nucleus or flagellum. It sometimes happens that a coverslip-film may be otherwise satisfactory, but may have dried slightly at or near the edges, thus affording opportunities for comparing the effects of desiccation on the trypanosomes with the condition of others that have never been dried. It is then seen that, in addition to the defective staining already described, the trypanosomes are flattened and distorted in various ways.

The fragments of tissue in the dissection adhere, for the most part, to the coverslip; it is not possible, however, to make out anything of trypanosomes which remain within the organs in film-preparations, and it is therefore necessary to tease up the organs well, after dissecting them out, in order to set free the trypanosomes. In the case of those phases which are attached to the gut-wall many remain so attached even when the wall is teased up, but a certain number are usually set free. When such forms are seen in the fresh film they should be dislodged, as far as possible, by tapping gently on the coverslip with a needle.

In some cases a coverslip-film which had been stained with iron-hæmatoxylin was unmounted by dissolving the Canada balsam in xylol, after the trypanosomes on it had been

studied and drawn, and the hæmatoxylin-stain completely extracted by placing it for twenty-four hours in a 3½ per cent. solution of iron-alum. The coverslip was then washed for an hour in a current of tap-water, and could then be re-stained by some other method—for example, Twort's stain. Trypanosomes that had been already drawn after the hæmatoxylin-staining could then be drawn again after being stained in a different manner. This double staining did not seem to injure the trypanosomes in any way, but it is noteworthy that after re-staining with Twort's stain they always came out a little smaller, when re-drawn with the camera lucida, than they had done previously after the hæmatoxylin-stain (compare figs. 260–63, Pl. 42, with figs. 260a–263a, Pl. 38).

When, as sometimes happened, the trypanosomes were so scanty on the coverslip as to require prolonged searching to find them, it was often very difficult to judge the right amount of extraction of the hæmatoxylin in the process of differentiation by means of iron-alum. Moreover, a degree of differentiation which is sufficient for trypanosomes in the thinner parts of the film is insufficient for the thicker parts. Hence it was often necessary to unmount the preparations and differentiate them further, perhaps two or three times, before the right degree was attained. It is difficult to judge of the required differentiation by the fragments of tissue in the films, since the minute bodies of the flagellates give up the stain much more quickly than the relatively thick tissue-cells, and in a preparation in which the latter are satisfactorily differentiated the trypanosomes become mere ghosts, requiring to be re-stained altogether. The counter-stain with the Lichtgrün-picric mixture was found to show up the cytoplasm and flagellum of the trypanosomes more clearly.

However carefully the preparations have been made, it is often difficult to make out clearly and with certainty the structural details of some of the minuter phases of the life-cycle, and for this purpose the best optical apparatus was required, both as regards the objectives and the illumination

used. All trypanosomes in the permanent preparations were drawn by Miss Rhodes, under our supervision, with the camera lucida at a constant scale of magnification which was as nearly as possible 3000 diameters in the case of the film-preparations, 2000 diameters in the case of sections.

Our study of the development of *T. lewisi* in the flea was based principally upon the examination of films, made as described above, but it was found necessary also to cut sections both of the stomach and rectum of the flea. The following is an account of the technique employed by us in preparing sections of the stomach; the same applies to sections of the rectum, the only difference being that the stomachs were cut transversely, the recta longitudinally.

The stomachs of which sections were cut were taken from fleas fed eighteen, twenty-four, or thirty-six hours previously on an infected rat; the fleas themselves had been collected from the non-infected breeding-cage and kept hungry for about three days before being put on the infected rat. The stomach in each case was carefully dissected out from the flea, if possible without puncturing or injuring the stomach, in a drop of salt-solution on a slide, and then plunged into the fixative by inverting the slide in such a way that the stomach alone, all other parts of the flea having been removed, was in a hanging drop. If the stomach was ruptured or punctured in the process of extraction it was not, as a rule, preserved, except perhaps as a smear after teasing it up.

A number of different fixatives were tried, but the best results were obtained with Flemming's fluid¹ and Maier's modification of Schaudinn's fluid, and especially with the former. After Flemming the histology of the stomach is extremely good in all details; the blood fills the whole section

¹ The strong solution, made up as follows: a gramme tube of osmic acid is broken into a clean bottle, and to it is added distilled water, 50 c.c.; 1 per cent. solution of chromic acid in water, about 187.5 c.c.; and glacial acetic about 12.5 c.c.; the whole allowed to mix and dissolve.

and is not shrunk away from the wall, and the trypanosomes, both free and intracellular, are well-preserved both in structure and form, and they stain well either with iron-hæmatoxylin or Giemsa, especially the former. After Maier's fluid the histology of the stomach-tissue is not so good; the cells are shrunk and the minute structure of the nuclei is deformed. It is evident from a careful study of the preparations that the defects of Maier's fluid are due to unequal or differential penetration of its constituents; the alcohol evidently diffuses into the tissues first and produces the shrinkage and deformation of the nuclei; the sublimate does not get to the various tissue-elements until they have already been fixed in a defective manner by the alcohol. The blood-débris is also much shrunk after the Maier; while the greater part, sometimes the whole of it, contracts to form a central mass in the section, a certain amount remains usually adherent to the epithelium at the periphery, leaving an irregular empty ring-shaped space between the central and peripheral zones of the blood-débris. But to compensate for these disadvantages, the trypanosomes are extremely well-preserved and stain admirably with Giemsa's stain; some of our stomach-sections prepared in this way are as clear and demonstrative, so far as the trypanosomes are concerned, as any smear or film-preparation; in fact more so in the case of the large "spheres," which do not suffer so much from the tendency to opacity which is so disagreeable a feature in the smears. One is here confronted with the extraordinary difference, familiar to everyone who has worked at trypanosomes, between the reaction of these parasites, and that of tissue-cells, to the ordinary fixatives and stains used in cytological technique.

Whatever the fixative used, it was allowed to act for about an hour. The stomachs preserved in Flemming were well washed in tap-water and then brought up through a series of alcohols of gradually increasing strength; those preserved in Maier were transferred from it direct to 50 per cent. alcohol. In either case the objects were brought up to 90 per cent.

alcohol and there fixed on liver preparatory to being imbedded for section-cutting. Amyloid human liver was used. A moderately thin slice of a block of liver preserved in alcohol was cut by hand with a razor wetted with alcohol, and floated into a shallow glass vessel with a flat bottom, placed on the stage of the dissecting-microscope, and containing 90 per cent. alcohol to the depth of about a centimeter. The stomachs, taken up in a pipette of suitably coarse calibre, were placed on the slice of liver and carefully arranged side-by-side, their axes parallel to one another and similarly orientated, with their proventriculi all at the same level and all pointing in one direction, their pylori in the opposite direction. Then a tiny drop of glycerine and albumin solution, such as is used commonly for sticking sections on slides, was taken up on the point of a needle and caused to touch the surface of the alcohol immediately above the stomachs. The dense albumin-solution falls at once through the alcohol and spreads out over the stomachs on the liver; at the same time the glycerine is extracted and the albumin coagulated by the alcohol, with the result that the stomachs are stuck to the slice of liver. From six to nine stomachs were thus attached side-by-side on a slice of liver. As the stomachs, before being stuck on, are very liable to roll about or become shifted in position with the slightest disturbance or touch of the microscope, it was found best in practice to put them on not more than three at a time; that is to say, three stomachs having been arranged and fixed upon the liver, three more are then put on beside them. When the required number of stomachs have been stuck on, the slice of liver is trimmed with a scalpel into a rectangular form, in such a way that the longitudinal axes of the stomachs are parallel to the shorter sides of the rectangle; so that by cutting sections of the liver parallel to the longer sides of the rectangle the stomachs are all cut transversely at the same time.

We have thought it worth while to describe the method of fixing the stomachs on liver, although no novelty is

claimed for it,¹ in some detail, as it may not be familiar to some investigators working on similar objects, and because it is a procedure which saves much time and trouble. In the first place, it is much easier to imbed a relatively large block of tissue than a number of separate tiny little stomachs, and the orientation of the objects can be made much more accurate. In the second place, a great economy of labour in the section-cutting and of space in the slides and preparations is effected. To have a number of stomachs cut in the same section diminishes the labour of looking through the preparations under the microscope, and the presence in the section of the slice of liver makes it much easier to go from one section to the next under the high power. Thirdly, with a little experience the liver itself furnishes useful guidance in staining the sections, especially by the iron-haematoxylin method; one soon learns what degree of extraction of the stain from the liver-cells gives the best results for the trypanosomes, so that the process of differentiation can be carried out under low powers of the microscope—a great advantage. And finally, since it may be assumed that all the stomach-sections contained in one and the same microtome-section have received exactly the same treatment, it is legitimate to ascribe the very considerable differences seen in different stomachs in the same section to constitutional or functional differences in the stomachs themselves and not to varying local effects of the stain.

The stomachs, after being fixed to the liver in 90 per cent. alcohol, were imbedded in the usual way in paraffin, with a melting point of about 54° C. Methods of celloidin-imbedding were tried, but yielded no advantages to compensate for the extra trouble, especially that of extracting the celloidin from the sections—an indispensable preliminary to staining them. The best thickness for the sections of stomachs was found to be 6 μ ; with less than that the trypanosomes are too

¹ One of us (E. A. M.) first became acquainted with this method in 1891 from fellow-workers in the Zoological Station at Naples, and has practised it constantly ever since.

fragmentary. The recta may with advantage be cut thinner than 6μ , since the crithidial forms are very minute.

Various methods of staining were tried on the sections, but the results of the trials were that we kept finally in practice to two methods only, namely, iron-hæmatoxylin (Heidenhain), followed by Lichtgrün-picric in absolute alcohol as a counter-stain, and Giemsa's method. For the iron-hæmatoxylin method the sections were treated first as has been described above for the coverslip-films. The Lichtgrün-picric, which stains very rapidly, was merely washed over the sections for a moment and then washed off again with absolute alcohol. Giemsa's stain was used, according to the published prescription, as follows: The sections have their paraffin removed, and are brought down to water in the ordinary way. They are then washed in tap-water and put into dilute Lugol's solution (1 c.c. of Lugol to 25 c.c. of distilled water) for ten minutes. After this they are rinsed quickly in tap-water and put into a 0.5 per cent. watery solution of hyposulphite of soda for ten minutes. Next they are washed in a current of tap-water for five minutes or longer, and then put into the stain. The distilled water used to dilute the Giemsa-stain has to be neutralised in the way prescribed by Giemsa.¹ The sections were first placed in fairly strong stain—say, 1 drop of Giemsa to 1 c.c. of neutralised distilled-water—for about an hour, and then were left overnight in a weaker stain—1 drop of Giemsa to 4 or 5 c.c. of neutral distilled-water. The excess of stain is removed by rinsing in water, and after the excess of water has been drained off differentiation of the stain is carried out with

¹ A measured volume of the distilled water to be neutralised is taken, and to it are added a few drops of hæmatoxylin-solution (5 per cent. in distilled water), sufficient to tint it. Then a very weak solution (1 per cent. in distilled water) of potassium carbonate is added drop by drop, the water being well shaken after each drop has been added, and left for a minute or two, until the colour of the tinted water changes from yellowish-red to reddish-purple. In this way the number of drops of the carbonate-solution required for neutralising a given volume of the distilled water is known.

different strengths of acetone mixed with xylol, beginning with 95 per cent. acetone used for a very short time, in order to dehydrate the sections and extract the stain, and ending with pure xylol, after which the slides are mounted in dammar or Canada-balsam.

Of the two staining methods principally used, iron-hæmatoxylin gave admirable results after Flemming, especially for the intracellular stages; for the extracellular trypanosomes this stain is not so satisfactory, owing to the fact that the blood-débris, especially in the earlier stages of digestion, stains very intensely with it and refuses to give up the stain—at any rate not until after it has been all extracted from the cells and parasites. Consequently trypanosomes free in the blood may be entirely obscured by the opaque, deeply-stained débris, and hence quite invisible. The black stain of the blood-débris is even more intense after Maier than after Flemming; sections of stomachs fixed in Maier less than thirty-six hours after feeding are hopeless for the iron-hæmatoxylin stain, so far as the free trypanosomes are concerned, and those fixed in Flemming are not much better. By Giemsa's method, on the other hand, the trypanosomes in blood-débris are sharply differentiated and admirably shown; in the cells they are also good, better, perhaps, as "show" preparations, but not so precise in minute cytological details as by the iron-hæmatoxylin method.

To sum up the results of our experience in the technique of stomach-sections, we recommend: (1) Flemming's fluid, followed by iron-hæmatoxylin and Lichtgrün-picric; and (2) Maier's fluid, followed by Giemsa's stain. These two methods, supplementing each other, may be relied upon to reveal all essential details of the intimate life of the trypanosome and of the disturbances produced by it in the tissues of the host.

PART II.—THE DEVELOPMENT OF *TRYPANOSOMA LEWISI* IN THE FLEA.

(1) GENERAL INTRODUCTION.

From the results of experiments, described further below, it is shown that fleas fed on rats infected with *Trypanosoma lewisi* do not become infective to rats again until a period of at least five or six days has elapsed from the time that the fleas first ingested blood containing trypanosomes. From these experimental data it may be inferred that the developmental cycle of *T. lewisi* in the flea requires a minimum of five days for its complete course. The conclusions drawn from the experiments are confirmed by direct observation, since it is found, as will be described presently, that the little stumpy trypanosome which is the final form of the development in the flea, makes its first appearance in the rectum of the flea about five days after the development begins.

During the entire course of its development the trypanosome is confined to the alimentary canal proper of the flea, and is found in the stomach, intestine, and rectum; it is never found in the body-cavity (hæmocœle), and by a series of observations and experiments, which in our opinion are exhaustive (see below), we have convinced ourselves that the trypanosome does not penetrate into the salivary glands. It may, however, occur in the Malpighian tubules exceptionally, as the small crithidial form, characteristic of the rectal phase, attached to the wall of the tubes at or near their proximal opening into the proctodæum.

The developmental cycle can be divided conveniently into phases characteristic of the parts of the gut in which the trypanosomes are found, and we can thus distinguish a stomach-phase and a rectal phase. These distinctions are useful and natural, but their sharpness is blurred by not infrequent variations in the course of events; thus forms belonging normally to the rectal phase may sometimes be found in the

pyloric region of the stomach, though the converse case of the typical stomach-phase occurring in the rectum is not found. We may consider these phases first in their normal and typical modes of occurrence, and deal with the variations subsequently.

The stomach-phase (Pls. 36-39) is the first period of the development and is characterised by a peculiar mode of multiplication on the part of the trypanosomes, which penetrate into the epithelial cells lining the stomach and there reproduce themselves by a process of multiple fission. Hence in this period of the development free and intracellular forms can be distinguished. The stomach-phase is of short duration, perhaps in some cases lasting not more than twenty-four hours, in others two or three days, in rare cases four or even five days, but probably always terminated by the second feed of the flea, counting as the first feed that by which the flea became infected.

In the intestine the trypanosomes find, as a rule, no resting place, but merely pass through it on their way to the rectum. Hence, the forms found in the intestine are usually active, migratory forms which have completed the stomach-phase and are on their way to the rectum to initiate the rectal phase. Occasionally, however, forms similar to those characteristic of the rectal phase may be found attached to the wall of the intestine, especially near the pyloric opening.

The rectal phase (Pls. 41 and 42) consists chiefly of small, often minute individuals, which are crithidial in structure and are attached by the tip of the flagellum to the wall of the rectum, where they keep up a continual multiplication by binary fission. The crithidial form of the development takes origin in the rectum and is first established there, but may migrate forward to the pyloric region of the stomach later on. When once established in the flea, the crithidial phase endures, probably, as long as the flea lives, and thus constitutes a permanent stock of the parasite, enabling the infectivity of the flea to be maintained without renewal of the infection. From the crithidial phase arise by modification of individual

crithidial forms the small trypanosome-forms by which the infection of the rat is brought about, and which are the final forms of the developmental cycle in the flea.

By no means all the trypanosomes, however, which are taken up from the rat by the flea undergo the course of development sketched out briefly in the foregoing paragraphs. By experiment it is found that only a relatively small number of the fleas fed on infected rats become infective, apparently not more than one flea in four, on an average (see below); and these results are confirmed by direct observation. If a number of fleas are fed on a well-infected rat, trypanosomes will be found in the gut of all the fleas dissected and examined a short time after feeding; but the longer the interval between the feeding and the examination of the fleas the larger the proportion of the fleas in which the trypanosomes have disappeared or become very scanty, until finally trypanosomes will be found in but few (see Tables A and B (2) above). Probably the percentage of fleas in which the trypanosome succeeds in establishing itself permanently may be taken, on the average as about 25 per cent. (see p. 663 below). It follows that in about 75 per cent. of the fleas which digest blood containing *T. lewisi* the parasites die out altogether, and it is probable that in all the fleas a certain number of the ingested trypanosomes die off, since fleas that have been fed on a rat with trypanosomes swarming in the blood may exhibit a very scanty infection of the gut at any subsequent period.

From these data it is to be expected that together with developmental forms of the trypanosomes, various stages in their degeneration would also be found in the fleas, at least during the first few days after the parasites were ingested by them, and this expectation is fully realised. It is necessary, therefore, to recognise a degenerative series of forms (Pl. 43) as well as a developmental series in the gut of the flea, and to distinguish carefully the two series from one another. Any particular flea, when dissected and examined, may present an extraordinary medley of different forms of the trypanosome. To distinguish between the different forms and to refer each

form to its proper position and sequence in the series, whether developmental or degenerative, is our task, and it is no light one. When we were at an earlier stage in our investigations we did not recognise sufficiently the importance of the degenerative series, and consequently tried to interpolate degenerative forms into the developmental series, greatly to our own confusion. On the other hand it is necessary to steer very clear of a tendency to explain any form as degenerative, of which the developmental position is not immediately clear; thus we were at first inclined to regard the peculiar recurved forms in the stomach as degenerative, until we discovered the intracellular multiplication and were thereby enabled to refer the recurved forms to their true position.

In the problem of piecing together and reconstructing the sequence of the two series, developmental or degenerative, there is, to begin with, a known and fixed starting point for each, namely, the ordinary form of *T. lewisi* as it occurs in the blood of the rat. Further clues are obtained by linking together, through gradual transitions, the forms seen in the fleas, but more especially by the study of "time-fed" fleas, that is to say, fleas dissected and examined at known periods of time after they have been fed on the infected rat. The part of the gut in which a given form occurs is a further guide as to its significance; and all data and conclusions obtained from observation are controlled and checked by the results of experiment, especially useful in determining the final form of the development. Guided by these various considerations we have arrived at the conception, set forth below in fuller detail, of the changes undergone by the trypanosome in the flea (see especially Pl. 45 and description). In our account we describe separately the two series which we regard as developmental and degenerative respectively; but it must be pointed out that while these two series are very distinct and easily recognisable as a whole, certain forms or stages of the one series are sometimes very difficult to distinguish decisively from very similar forms belonging in reality to the other series. Consequently it is impossible to

be free from doubt, occasionally, with regard to the place to be assigned to a particular specimen or type of individual.

It remains only to be stated at this point that we adhere to the following nomenclature for the parts of the body of the trypanosome or crithidia: Blepharoplast for the basal granule of the flagellum; kinetonucleus for the smaller, trophonucleus for the larger, of the two nuclei. In order to save space we shall, however, use for the kinetonucleus the symbol *n* (plural *nn*) and for the trophonucleus the symbol *N* (plural *NN*).

(2) THE DEVELOPMENTAL SERIES.

(A) The Stomach-Phase.

The blood ingested by the flea passes in the first instance into the stomach, that portion of the digestive tract which is derived from the embryonic mid-gut or mesenteron, and which is lined by a layer of epithelium representing the true hypoblast or endoderm of the embryo. In the post-embryonic stages of the insect, this part of the gut is characterised by the absence of the chitinous cuticular lining secreted by the ectodermal epithelium of the parts anterior or posterior to it, namely, the stomodæum, comprising the pharynx, œsophagus, and proventriculus, and the proctodæum, comprising the intestine and rectum. The boundary between mid-gut and hind-gut is further indicated by the origin at this point of the Malpighian tubules.

In what may be called a normal feed, the flea fills the stomach and proventriculus alone. It is not an infrequent occurrence, however, for some fleas to gorge themselves to such an extent that the freshly ingested blood not only fills the stomach completely, but overflows beyond it into the intestine and rectum; we have observed this to happen most frequently in the case of female fleas, rarely in the case of males. In such cases some of the ingested trypanosomes

may be carried on at once into the proctodæal regions of the gut, but all such trypanosomes degenerate, and need not be reckoned with in the developmental series, the first phases of which take place always in the stomach alone.

(a) The Extracellular Trypanosomes.

The trypanosomes introduced into the stomach very soon begin to undergo changes (Pl. 36, figs. 1 and 2; Pl. 37, figs. 47 and 48). The first change is probably purely physiological, since long before any alteration is observable in form or structure these ingested trypanosomes seem to have lost their power to infect when injected subcutaneously into clean, susceptible rats (see below, p. 634). The next change observed in these ingested trypanosomes may be seen on examining microscopically the contents of a flea's stomach four to six hours after the first feed on an infected rat. A certain number of trypanosomes will then be seen to pass rapidly in a straight course across the field of the microscope with their flagella directed anteriorly. The posterior third of the body is held more or less straight and appears more rigid, as it does not share in the rapid undulations of the anterior end of the body. The movements of these trypanosomes thus contrast strongly with the sinuous, serpentine and wriggling rather than progressive movements characteristic of the trypanosomes in the blood. When not actively progressing, the trypanosomes in the stomach have a tendency to attach themselves by the tips of their flagella to pieces of débris, to the wall of the stomach, or to the surface of any other firm body. The stiffening of the trypanosome-body is probably due to increased tension of the cytoplasmic contents produced by absorption of fluid from the ingested blood as it undergoes alteration in the process of digestion. As a result of absorption or imbibition of fluid, the body of the parasite, previously more or less distinctly flattened, acquires a cylindrical and more rigid contour. If this explanation be correct, it follows that the first stimulus to developmental change is

to be ascribed to differences of osmotic tension in the fluid medium, as has been shown experimentally by Miss Robertson (1911) to be the case in the development of the trypanosomes of fishes.

In stained preparations most of the ingested trypanosomes show at first little modification from the ordinary blood-trypanosomes. In rare instances the nuclei may be approximated (Pl. 36, fig. 2). Some show a darker staining-reaction of the posterior third of the body, which appears, from the backward position of *n* in such forms, to be a sign of degeneration beginning to set in (compare Pl. 43, figs. 289, 290).

In their free active state the trypanosomes in the stomach are never found to be undergoing multiplication by any form of fission, and it is doubtful if they undergo any developmental changes further than those described above, until after they have multiplied within the cells of the lining epithelium of the stomach. The multiplication of the stomach-phase takes place solely within these cells, and although, in strict chronological order, we should now describe the intracellular stages of multiplication, it is more convenient for purposes of description to divide the stomach-phase into "free" and "intracellular" stages, and to describe all the free developmental forms before describing the intracellular multiplication.

It can be established by direct observation that the process of intracellular multiplication produces a long, free type of trypanosome which may be characterised by the term "crithidiomorph," because while externally similar in form and movements to a large crithidial type of flagellate, it lacks, as a rule, the diagnostic structural feature of a true crithidial form, since only exceptionally is *n* found actually beside or in front of *N* (Pl. 36, figs. 3-11, Pl. 37, figs. 49-57). We shall now proceed to describe in more detail this late, free form of trypanosome. It must, of course, be understood that as several generations of the intracellular stage may follow each other in succession (see below), free and intracellular forms in all stages of development can be found

together in the same stomach. We believe, however, that the typical crithidiomorph type of trypanosome always follows an intracellular stage, that in its less developed form it is the direct product of intracellular multiplication, and that though in this form it may again enter an epithelial cell and multiply, it is, in the more advanced form, the highest developmental type of the stomach-phase and is destined to pass down the intestine into the rectum where, after undergoing further modification, it initiates the characteristic crithidial rectal phase to be described below.

In the living condition the crithidiomorph form progresses at a great pace in a straight line with the flagellum directed anteriorly ("mouvement en flèche"), in much the same manner as does the early stomach-form above described. It is, however, considerably longer and the posterior end is more rigid and swollen, often distinctly clubbed. Owing to the rapid motion and imperfectly straight body, the clubbed appearance is exaggerated in the living condition, but stained preparations also show that some of the trypanosomes are distinctly clubbed in shape. Like the early stomach-form, when not actively progressing the crithidiomorph type has a strong tendency to attach itself by the tip of its flagellum to cells or débris, etc. Apart from its size the distinguishing characteristic between this and the earlier form is the marked approximation of the two nuclei, best seen in stained preparations (Pl. 36, figs. 7, 8, 10; Pl. 37, figs. 50, 51).

It has been mentioned that while, as a rule, the long, free stomach-trypanosomes have *n* behind *N*, it is found in a few cases that they have the typical crithidial structure with *n* in front of *N*. We have observed altogether but three instances in which such forms occurred in sufficient abundance to make them worthy of special note. The first and most striking was the case of a flea taken from a bell-jar in which a number of fleas had been kept for some time with an infected rat, so that the length of time since the flea had ingested the parasites was not known. The body-cavity of the flea contained a cysticeroid of *Hymenolepis diminuta* (vide Nicoll and Minchin, 1911). The intestine of this flea showed a peculiar malformation in the form of a globular pouch-like appendix, distended with red fluid, and due apparently to an obstruc-

tion or strangulation of the intestine. The stomach, examined fresh, was seen to contain a great number of active trypanosomes, some of which were adhering together in couples, and in the intestine a clump of attached forms was seen near the origin of the Malpighian tubules.

In the preparations of the stomach of this flea a great number of trypanosomes were found showing every possible gradation of structure, from forms similar to the ordinary blood-trypanosomes to a long crithidial type with *n* far in front of *N* (Pl. 36, fig. 11 and Pl. 37, figs. 60-66). Many of these were found closely adhering in couples, just as had been seen in the fresh state, each such couple being composed of two crithidial forms in most cases, but sometimes of two ordinary forms (Pl. 36, fig. 12, and Pl. 37, figs. 67, 68). In every couple seen the two individuals appeared quite distinct and showed no signs of actual fusion; one couple was found attached tête bêche (as in Pl. 43, fig. 310). In the preparation of the rectum and intestine (preserved together) a few similar large trypaniform or crithidial individuals were seen, and also a fair number of dwarfed, degenerative forms, but no couples.

Special mention has been made of this flea because we were at first, and remained for some time, under the impression that the couples seen represented a true sexual fusion, and that we had discovered the sexual phase of the trypanosome. We have been quite unable, however, to confirm this notion or to find a similar state of things in any other flea of all those examined by us, and we now regard the state of things found by us in this particular flea as exceptional and abnormal, in relation probably to the malformations noted by us in the flea itself. It is possible that the malformed condition of the intestine prevented, to some extent, the passage onwards of the trypanosomes from the stomach, and so caused an arrest of development in the parasites, in which the tendency towards the crithidial type of structure became realised to its fullest extent. The coupling of the trypanosomes must then be regarded as agglomeration due to abnormal and unfavourable conditions, though in no case were more than two trypanosomes seen adhering together.

The second case in which the long crithidial forms were prominent was in a flea of a batch which had been fed on an infected rat three days before being examined and dissected. The fleas had been kept in an incubator at a temperature of 25° C. after the infective feed, and had not been fed again. In one of the fleas long crithidial forms with *n* in front of *N*, were fairly numerous, together with intracellular multiplicative stages, in the stomach (Pl. 37, figs. 56, 57); in the rectum one active trypanosome of the long stomach-type and a clump of degenerative forms were seen in the fresh state.

The third case to be noted was in a flea of a batch which had been fed twenty-four hours previously on an infected rat. There was nothing

special to note about this flea; the stomach contained many long active trypanosomes, with n and N closely approximated, or with n in front of N (Pl. 36, figs. 9, 10), and also some dwarfed degenerative forms, but no multiplicative stages. In the rectum a few clumps of degenerative appearance and some developmental forms were seen.

Besides these three cases which have come under our observation, in which the long crithidial type was conspicuously abundant in the stomach, we have noted the occasional occurrence of this type at various ages—twenty-four hours, forty-eight hours, and sixty hours—after the flea had fed on an infected rat. It is evident that it must be regarded as exceptional for the trypanosomes to reach the complete crithidial condition in the stomach, and that no special significance can be attributed to the crithidial form in this part of the life-cycle, although in rare instances and under special circumstances it may be abundant.

It will be clear from the foregoing remarks that we are quite unable to agree with the statements of Swellengrebel and Strickland (1910), who, having examined two fleas one day after feeding on the infected rat and five others two days after the infected feed, describe the transformation of the long crithidiomorphic type of trypanosome into the long crithidial type as the normal and usual method of development in the stomach. On the strength of somewhat more extended experience, we consider the long crithidial form to be of highly exceptional occurrence, both in the stomach and elsewhere, at so early a period of the development, as already stated: we can only explain the results of Swellengrebel and Strickland on the supposition that they were so unfortunate as to have chanced upon abnormal fleas, similar to the three cases described by us above, or that they may have regarded as crithidial forms the very commonly-occurring recurved forms, which they do not describe at all.

In view of what is known with regard to both the later development of *T. lewisi* in the flea and the life-cycle of other trypanosomes in their invertebrate hosts, it is evident that the crithidial type of form and structure is the principal and most characteristic phase of the development, and that there is a pronounced tendency for the trypanosome to assume crithidial characters when taken up by the flea—a tendency which asserts itself more strongly after the trypanosomes have undergone multiplication in the cells of the lining epithelium of the stomach. So long, however, as the trypanosome remains in the stomach the atavistic tendency towards

the assumption of the crithidial form $\left(\frac{n}{N}\right)$ does not normally (or, at all events, usually) get beyond the crithidiomorph form $\left(\frac{N}{n}\right)$. Occasionally, nevertheless, the crithidial form asserts itself, as it were, even during the stomach-phase; more especially, perhaps, under the influence of any circumstances which tend to retard the development of the trypanosome and retain it in the stomach after it is ripe for passage into the proctodæum, but not infrequently even under conditions which cannot be asserted to be in any way abnormal.

(b) The Intracellular Multiplication of the
Trypanosome.

As already stated, the multiplication never takes place in the free, active condition of the trypanosome, but only after it has penetrated into one of the large epithelial cells lining the stomach, within which it goes through a process of multiple fission to produce a number of daughter-individuals which escape from the cell and pass back into the lumen of the stomach as free trypanosomes again. The whole process of intracellular multiplication, so far as it could be made out by observation of living trypanosomes in the stomachs of freshly-dissected fleas, was described by us in our preliminary report (1911); we had not then had sufficient time or opportunity to make detailed studies, which present peculiar difficulties, of the multiplication in preserved and stained material. The ordinary smear-methods seldom permit any finer details to be made out of the trypanosomes within the cells, on account of the large size and thickness of the cells and consequent opacity of the preparation. It is only possible in smears to study the stages of multiplication set free by the rupture of the cells; but even of such specimens it is difficult to get perfectly satisfactory preparation for microscopic study of detail. With the method of fixation by vapour of osmic acid and subsequent coloration with Giemsa's stain or other

modification of the Romanowsky method of staining we have obtained occasionally very clear preparations of the later stages of the multiplication, but as a rule, the "spheres" with many nuclei take up the stain with such intensity that they become opaque masses showing nothing of the internal structure, although in the same preparations the free trypanosomes may be stained to perfection. If in such preparations the stain be cautiously extracted by means of acetone or other suitable media, it is possible to obtain specimens showing the nuclei satisfactorily, but then, as a rule, the flagella are invisible, having lost the stain completely, while the free trypanosomes or early stages of multiplication on the same slide have become mere ghosts or have vanished altogether, beyond the power of visual resuscitation by the most delicate and refined methods of microscopic illumination. Very often in such preparations only the kinetonuclei can be seen, the trophonuclei having disappeared. In the study of Romanowsky-stained preparations it was generally found necessary to begin by drawing all that could be seen, general outline, projecting flagella, in the opaque, untouched preparations of the spheres, and then to perform a number of successive operations of cautious extraction of the stain, examining the preparations after each such operation and adding to the drawing any fresh details of structure brought to light. It was difficult, however, to control the extraction of so sensitive a stain with sufficient exactness to avoid losing the whole of it in an instant. The last state of the preparation was generally one which left it useless for purposes of demonstration; always a disappointment to the microscopist and his friends. We have never succeeded in re-staining satisfactorily preparations in which the Romanowsky stain has been over-extracted.

By far the best and most instructive preparations of the intracellular multiplication were obtained in the coverslip preparations fixed in Schaudinn's or Maier's fluid and subsequently stained by the iron-hæmatoxylin method, as described above. Only in such preparations was it found possible to

control the stain so that in the largest spheres both nuclei and flagella were visible; even then, however, the tropho-nuclei were sometimes faint and difficult to make out clearly when the flagella were still sharp and distinct. Of one film in which the smear was thickly crowded with spheres of various sizes, some free, others still in the tissue, a very satisfactory preparation was obtained by staining with Mann's hæmatoxylin, carried out with the friendly help of the inventor of the stain himself. The result was a very good "show" preparation of the multinucleate spheres, sharp and clear, even in the thick parts of the smear, and especially suitable for moderate magnification; the flagella, however, could not be made out.

While the ordinary smear-methods presented special difficulties, very convincing and beautiful preparations of the intra-cellular phase were obtained in sections of fleas' stomachs extracted carefully from the body and preserved in various ways; a full account of the technique employed is given above. Such preparations have the immense advantage of exhibiting the exact relations of the trypanosomes to the cells; it is possible to look through every section of each series, to note every trypanosome, free or intracellular, occurring in each stomach, and to observe what each parasite was doing at the moment the stomach was preserved. On the other hand, for the study of the stages of multiplication, sections have the disadvantage that the parasites themselves are often halved or mutilated, so that any given specimen may be only a part or fragment of the whole body. Both smears and sections are therefore indispensable, and supplement each other in obtaining a complete picture of the course of events.

So much for methods and technique; we proceed now to give an account of our observations.

From Nöller's investigations on the development of *T. lewisi* in *Ctenocephalus canis*, it appears that the intracellular multiplication begins about six hours after the ingestion of the trypanosomes by the flea. In our prepara-

tions of a batch of fleas, of which the infection could not have been more than nine and a half or less than seven and a half hours old, we have found the recurved forms fairly commonly and also some of the rolled-up forms characteristic of the early intracellular stages (Text-fig. 23, p. 635). We have no other records of intracellular multiplication in our fleas earlier than twelve hours. The stages of the intracellular multiplication are to be found in all parts of the epithelium of the stomach, from close behind the proventriculus to the pylorus.

Our investigations upon the intracellular multiplication contain, unfortunately, one gap which we have been unable to fill; we have not succeeded in observing the actual penetration of the epithelial cell by the trypanosome. Nöller, however, has been so fortunate as to observe the process, and gives the following account of it. In a dog-flea which had sucked infected blood five hours and fifty-five minutes previously, he saw "a trypanosome, of which the pointed hinder end had already penetrated into an epithelial cell. The flagellum-bearing anterior end beat violently and incessantly, whereby the trypanosome penetrated further and further into the cell. After I had watched this spectacle for about five minutes the trypanosome, which had so far penetrated into the cell as far as the middle of its body, shot suddenly into the cell and stirred up the granular cell-contents by its lively movements. Since, however, the cell was torn on the opposite side, the trypanosome soon shot out of the cell again." Nöller thus confirms the suggestions we made in our preliminary report (1911) with regard to the probable method in which the penetration of the cell is affected.

We have frequently seen trypanosomes, not distinguishable in the living state from the ordinary type, singly within cells; the first time we ever discovered the trypanosomes within the cells was just such a case, a single trypanosome of quite ordinary appearance, wriggling and squirming actively in the cytoplasm of an epithelial cell, in a flea which had

been fed twelve hours previously on an infected rat. Careful examination of the cell, at different foci of the microscope, convinced us, greatly to our astonishment, that the parasite was really within the cell and not above or below it. This, and other observations, repeated subsequently upon trypanosomes of ordinary appearance, contained singly within epithelial cells, suggest that the trypanosomes in each such case had but recently penetrated into the cell; but the observation might also be interpreted to mean that the trypanosome seen was the last of a batch produced by multiple fission within the cell from which its sister-trypanosomes had already escaped. In the latter case, however, the trypanosome would probably be within a vacuole, as will be described presently.

Observation of the free trypanosomes in the living state shows that, as already stated, they are extremely active, but have a great tendency to attach themselves by the tip of the flagellum to firm objects; to the wall of the stomach, to pieces of débris, even to the glass surface of the slide or coverslip when under observation. The study of sections of the stomach confirms this observation in an unmistakable manner; many trypanosomes of the long, stiff type are seen in the sections attached to the epithelial cells by their flagella. The attachment is not, as a rule, to the outer projecting ends of the cells, but to their sides; the trypanosomes put their long flagella down between the epithelial cells and often adhere to the cell close to its base; it would appear as if the side of the cell, at least in its columnar form, is its vulnerable region. A still more striking point is that many of these trypanosomes attached to, but still quite outside the cell, have already assumed the recurved form. These observations make it very probable that in some cases the trypanosome may first attach itself to the cell by its flagellum and then bore its way into the cytoplasm in some way.

Several trypanosomes may penetrate independently into one and the same cell. We have frequently observed numbers of the parasites, from five or six up to a dozen or

more, in different stages of multiplication, side by side in a cell both in the living condition and in sections (Pl. 38, figs. 112, 113; Pl. 39, fig. 130). The parasites lie in the cytoplasm usually in a distinct vacuole, produced, apparently, by the liquefactive action of the parasite on the cytoplasm of the host-cell. The trypanosomes are nearly always in a state of movement, a point to which we shall return again. It is not infrequent to observe a number of trypanosomes in the same vacuole, wriggling actively one over the other.

The infected cell may become reduced simply to a bag containing fluid in which large numbers of trypanosomes, generally with their multiplication completed or far advanced, move actively. Such cells are found commonly in sections (Text-fig. 3); they are generally thrown off from the epithelium and lie quite free in the blood-débris, sometimes even in the centre of the lumen of the stomach; nothing remains of the cell-contents except a thin superficial layer of cytoplasm, under the cell-membrane, and the nucleus, adherent usually to the wall at some point. This condition obviously represents the last stages of the exhaustion and death of the cell, from which the trypanosomes will escape either by their own activity or by disintegration of the cell. We shall consider the effects produced by the parasites on the cells in more detail subsequently; at present it will be more convenient to confine our attention to the development of the trypanosomes themselves.

The study of the trypanosomes in the living cells, checked by the examination of preserved material, permits readily enough of the recognition of a number of well-marked stages in the process of multiplication:

(1) Trypanosomes of quite ordinary appearance, which have apparently but recently penetrated into the cell, as already described.

(2) Pear-shaped forms, with the flagellum continuing the stalk of the pear; the body of the pear is distinctly flattened, and therefore presents a contour which differs according as it is seen from the edge or from the flattened surface; as the

parasite is in constant motion within the cell it presents continually different views to the observer. The body of the parasite also shows in life incessant "metabolic" changes of form, movements of an active protoplasmic body imperfectly restrained by the thin, yielding envelope or periplast. It is very easy to see in the living condition that these pear-shaped forms are produced by the body of the trypanosome being doubled upon itself, an interpretation confirmed by the examination of preserved specimens. When the stomach is teased up and examined fresh, many of the recurved pear-shaped forms are found swimming freely, with the flagellum forward, in the salt-citrate solution used in the dissection. If such a form be watched attentively, it is often seen to uncurl itself, straightening out the body and thus passing from the pear-shaped form to that of an ordinary trypanosome. In some cases a trypanosome which had been seen to unbend itself in this manner can be observed to curl up again, while swimming freely, and thus to assume or to lose the pear-shaped form several times in succession.

In the fixed and stained preparations it is seen that the trypanosome is bent upon itself in such a way that the posterior part of the body, containing the kinetonucleus, is closely applied to the anterior half of the body. Thus a pear-shaped body results in which *N* is lodged in the thickest part of the pear, at (Pl. 36, fig. 15) or near (fig. 13) the blunt end of the body; while *n* is usually well in front of *N*, that is to say, nearer to the pointed end of the body (Pl. 37, fig. 70). In some cases, however, *n* is close beside *N* (figs. 13-17), or even, exceptionally, behind *N* (fig. 16). The variations in the positions of *n* and *N* are easily explained by their variability in this respect, already described, in the free trypanosomes, on the one hand, and on the other by variations in the exact region of the body at which the bending takes place. As a general rule, the body appears to be bent between *n* and *N*, so that *N* lies a little way from the extreme posterior end and *n* in front of it (figs. 13, 17); but the point of greatest curvature may be in the region of *N*, which is

then at the hindermost extremity of the body (fig. 15), or even in the region of *n*, which is then at the extreme blunt end (fig. 16). In exceptional cases the bending takes place in front of *N*. In all cases the flagellum runs backwards along one side of the body, round the blunt posterior end, and forward, for a variable distance, to the basal granule or true blepharoplast situated close beside *n*. Thus the course of the flagellum, as a whole, may be compared to the letter **U** modified by making one arm of the letter much longer than the other.

In some cases the distinction between the two limbs of the recurved body can be seen plainly in the fixed specimens (Pl. 36, fig. 15; Pl. 37, fig. 70), but in other cases no line of demarcation can be made out, and the applied portions of the body appear to have formed completely into a compact pear-shaped mass, leading on to the stage next to be described.

The recurved forms differ remarkably in size, and from a comparison of these forms with one another, with the free trypanosomes, and with later stages of the intracellular development, there can be no doubt that the initial stages of the life of the trypanosomes within the cells is accompanied by a pronounced diminution in the size of the flagellates (see especially Pl. 36, figs. 13-17, and compare them with figs. 1-10 of free trypanosomes, and figs. 21-34 of later stages, on the same plate). How this shrinkage takes place it is difficult to say; probably the cytoplasm of the flagellate gives up a large amount of watery fluid and so diminishes in bulk, while becoming at the same time correspondingly denser in texture, a change which would account for the intensity with which the intracellular forms take up the stain and the consequent opacity which they acquire, as already noted. It is necessary, however, to exercise caution in estimating the size of the forms in preparations, since there is no doubt that they vary owing to differences in fixation. Thus, Pl. 36, fig. 14 shows a specimen from the same slide as fig. 13, but the former is from a part of the film which appeared to have dried before it was exposed to the action of the osmic vapour;

its large size, light colour and the elongation of *N* are all indications that the soft body had become flattened out by being dried before fixation. The specimens may also become deformed in other ways; Pl. 37, fig. 72 is probably to be explained as representing a recurved form in which the flagellum has become torn away from the side of the body and so projects freely from the rounded posterior end.

We were at first under the impression that the pear-shaped, recurved trypanosomes found free in teased-up stomachs examined fresh were forms that had been originally intracellular and had been set free by rupture of their host-cells. As stated above, however, examination of sections proved that these recurved forms may be extracellular in occurrence, attached to the epithelial cells or even free from them. It is evident, therefore, that the recurved form is not simply an adaptation to life within the confined limits of the cell.

(3) Forms with rounded or oval body, derived from the pear-shaped recurved forms by a further contraction and rolling up of the body; these are the forms which we described in our preliminary account as "block-like" since the body often shows during life irregular contours, changing continually owing to the active metabolic movement. The flagellum, which runs in a U-shaped course in the recurved forms, acquires now an additional bend (compare especially Text-fig. 23, p. 635, *h* and *i*; it usually runs round the outer contour of the rounded body and protrudes from it to a variable extent. In some cases a very considerable length of the flagellum is free (Pl. 37, figs. 77, 78), in other cases a very little (Pl. 36, fig. 28; Pl. 37, fig. 82), while in other cases again the flagellum is simply wrapped closely round the body (Pl. 36, figs. 29, 30, 35; Pl. 37, figs. 81, 84). The extreme length of the free flagellum seen in Pl. 37, fig. 76 is possibly due in part to its having become artificially detached from the body in the process of making the smear.

The data in the foregoing paragraph have been obtained chiefly from the study of preserved specimens. In the living condition this stage appears as a small, rounded or oval body

within the cell, usually in motion and showing a distinct flagellum. Sometimes, however, these bodies are quite motionless with no flagellum visible. In our preliminary communication (1911), we were unable to decide whether a flagellum was always present, and were prepared to admit that in some cases this stage might be a non-flagellated leishmania-like form. We had observed in one case that a body which had been for some time quiet and motionless within the host-cell became suddenly active, showing a distinct flagellum. In all our permanent preparations, however, whenever flagella can be made out in the other stages or in the free trypanosomes, they can be seen to be invariably present at this stage also, and there can be no doubt that the motionless forms are those in which the flagellum is wrapped round the body. We are now convinced that non-flagellated leishmanial forms do not occur. We have the impression that the rolled-up trypanosome can wrap its flagellum round the body and pass into a resting, quiescent condition for a time, after which it can become active again by uncurling and setting free its flagellum, or at least a certain length of it, probably never quite the whole length.

The body in this stage, as in the last, is actively metabolic, with constantly changing contours in life. When the rounded forms are set free by rupture of the host-cell they swim actively in the liquid, progressing with the flagellum directed forwards; they then resemble ordinary flagellate monads, and the observer might easily have the impression that he was watching some intruding flagellate derived from contamination of the salt-solution or from some extraneous source.

In a few rare instances the rolled-up forms have been found in preparations to exhibit a central perforation or fenestration (Pl. 36, figs. 24, 30, 34), evidently produced by the trypanosome curling itself round so as to leave a central space. This condition, when it occurs, is probably quite transitory, the plastic cell-body of the trypanosome fusing into a compact lump sooner or later.

It seems probable that some of the rolled-up forms degene-

rate at this stage and are absorbed; that is, at least, the only explanation we are able to offer for such minute forms as those shown in Pl. 36, figs. 43-45, which appear to be undergoing degeneration.

The smallest of the rounded forms have each a single kinetoneucleus, trophonucleus and flagellum. Now they begin to grow in size, with concomitant multiplication of their nuclei and formation of daughter-flagella. The division of these various parts appears to go on much as in other trypanosomes, independently, but more or less synchronously. The division of n may be slightly in advance of that of N , or slightly after it: thus, stages are found in which n and N appear to be both in the same stage of division (Pl. 37, fig. 79); or in which N appears to be in advance of n (Pl. 36, figs. 28 and 37); or with two distinct nn and N still in division (Pl. 37, figs. 80, 82); or finally with n and N both completely divided (Pl. 36, figs. 33, 36). The division of n is dependent on, or connected with, that of the basal granule or true blepharoplast, which may be regarded as representing the centriole or division-centre for n . The original flagellum does not divide, however, but remains attached to one of the daughter-blepharoplasts, from which it arises in close proximity to one of the daughter nn ; and from the other blepharoplast a new flagellum grows out, at first a very fine and delicate structure and consequently very difficult to make out clearly or with certainty in the opaque body. In many cases in which n is divided completely no second flagellum can be seen, but it would not be safe, in view of the difficulties of technique presented by these objects, to conclude in all such cases that the formation of the new flagellum had not begun, since in other cases a very delicate line can be seen plainly growing out from the daughter-blepharoplast—that is to say, from the blepharoplast other than that from which the original flagellum arises (Pl. 37, figs. 79-81). As a rule the daughter-flagella can be made out in the preparations stained with iron-haematoxylin, but not in those stained by Giemsa's method; in the latter case, as already mentioned, the body

usually stains so intensely as to obscure completely the delicate daughter-flagella, which are imbedded in the mass of the cytoplasm, while the original flagellum runs for the most part on the exterior of the body; and if the stain be extracted sufficiently to make the body clear, it comes out of the growing flagella and leaves them invisible.

We may infer, therefore, that as the division of the nuclei proceeds the formation and growth of new flagella follow hard upon the division of the blepharoplast and kinetonucleus, and that a new flagellum grows out from each blepharoplast, in close proximity to *n*, quite independently of the original or parent flagellum, which remains unaltered.

As a general rule the multiplication of the nuclei begins at the rolled-up stage, but this rule is by no means invariable. Sometimes the nuclei are found to have multiplied even before the trypansome has taken on the recurved form (Pl. 36, figs. 18-20). Such forms might possibly be specimens which, after becoming recurved, have straightened themselves out again, but their appearance is that of trypanosomes in which nuclear multiplication has begun before change of body-form. Attention must also be drawn to the peculiar elongated forms with 2 *nn* and 2 *NN*, such as Pl. 36, figs. 21-23; Pl. 37, fig. 74. At one time we were inclined to suspect that these forms, and also the unaltered trypanosomes with 2 *nn* and 2 *NN*, might be examples of fusion instead of multiplication (see below, p. 604); but we could find no definite evidence of there being fusions of two trypanosomes, and the fact that forms occur with 3 *nn* and 3 *NN* (fig. 18, pl. 36) makes it more probable that they are early stages of multiplication. On the other hand the possibility cannot be excluded that in some cases accidental and purely plasmogamic (non-sexual) fusion of intracellular stages may occur, and that such fusions may explain the enormous size of some of the spheres and later stages of multiplication (Pl. 36, fig. 42).

The multiplication of the nuclei proceeds apace, and the duplication of *n* and *N* is followed by a stage in which the

body contains 3 *nn* and 3 *NN* (Pl. 36, fig. 40¹; Pl. 37, fig. 84). This stage, which is of common occurrence, indicates that after the first division of *n* and *N* one of the daughter-nuclei in each case remains undivided, while the other divides again. This interpretation is supported by fig. 39, showing a specimen containing 3 *nn* and 2 *NN*, one of the latter being in process of division. A later stage is seen in fig. 38, in which both *n* and *N* have multiplied to four in number. The further stages of multiplication are not easy to follow in detail owing to the difficulty of making clear in one and the same specimen the *nn*, *NN*, and flagella, but from a study of various preparations there appears to be no reason to doubt that the *nn* and *NN* maintain their parallelism in division, and consequent equality of numbers, and that as the *nn*, or, to be more accurate, their blepharoplasts, divide, they continue to give rise to new flagella. Since the blepharoplasts are of different ages, as the result of successive divisions, the daughter-flagella given off from them are of different lengths; the original or parent flagellum remains, however, distinct and recognisable, both by its length, its superficial position, and the sharpness with which it stains (Pl. 37, fig. 92, etc.). In some cases the daughter-flagella also project freely from the surface of the body. This fact was observed in a living specimen, in which two small flagella were seen in addition to the principal flagellum, but it appeared to be a temporary condition, since later on only the principal flagellum could be seen, and still later that also disappeared. In one of our preparations also three daughter-flagella are seen in addition to the main flagellum, but unfortunately the specimen was so opaque that no details of internal structure, except the nuclei, could be made out.

With the growth in size and the multiplication of the nuclei the "block-like" stages pass by insensible gradations

¹ The specimen shown in fig. 40 appears to have become dried and flattened out before fixation; its size is altogether abnormal for this stage; compare fig. 38. Fig. 26, Pl. 36, also abnormally large, is from the same slide as fig. 40.

into: (4) The "spheres," a term used by us to denote the final stages of the intracellular multiplication. They appear in the fresh preparation as relatively large masses, more or less spherical in form, within the cytoplasm of the epithelial cells; they can be distinguished generally as "tailed" and "tail-less." At the very last the tail disappears in all cases, but this appendage, though commonly present in stages not fully grown, sometimes cannot be made out in specimens that do not appear to be mature. A comparison of fixed preparations and a consideration of facts already discussed in describing the "block-like" stage leaves no doubt but that the tail-less spheres are derived from those earlier stages which have the original flagellum wrapped round the body, the tailed spheres from those which have the flagellum free. It is, however, evident even in the living state that the tail represents more than the original flagellum, since it is often of considerable thickness at the base and tapers gradually to a point. Examination of stained preparations shows that the tail represents in early stages the anterior end of the body, with the flagellum, of the original trypanosome (Pl. 36, figs. 37, 39; Pl. 38, fig. 110); and that in later stages the daughter-flagella may grow out parallel to the original flagellum, and so contribute to the formation of the tail (Pl. 37, fig. 86; Pl. 38, figs. 108, 111). But in other cases the anterior end of the original trypanosome may be retracted into the main mass of the sphere, round which the flagellum is wrapped more or less completely; the sphere then either has no tail (Pl. 37, figs. 90-94) or, if a tail is present, it represents the flagellum alone (figs. 95, 110).

The appearance and behaviour of the spheres in the living condition have been described by us in our preliminary communication. They are in a state of incessant movement, due both to the activity of the flagellum or tail, when present, and to internal commotions. The movements of the flagellum cause them to rotate within the cell in a jerky, irregular manner. When there are several spheres within one cell they can be seen to push and bump against

the cell-nucleus or against one another, the impact of one sphere causing a movement in another, which shows that they come into actual contact. Internal causes of movement are the metabolic form-change, already described, in the earlier stages, and movements due to the independent motility of the daughter-trypanosomes in the ripe spheres.

Even in the living condition it is not difficult to make out that a process of multiplication is going on within the sphere. First the nuclei and flagella can be seen to have multiplied, though the details of the process are naturally not clear in the living specimen. Later it is seen that the whole contents of the sphere have divided up into a number of distinct daughter-trypanosomes, which are seen writhing and twisting over each other within the enveloping periplast like a bunch of eels in a sack. The independent movements of the contained trypanosomes make their separate individuality clearer in the living state than in the preserved specimen. After the daughter-trypanosomes have become fully distinct and separate from one another a number of changes are seen in the sphere. The tail, if it was present, disappears, and the body acquires a perfectly spherical contour; this can only mean that the original flagellum, distinct from the beginning, has been drawn into the sphere and taken over by one of the sister-trypanosomes resulting from the process of multiplication. The metabolic form-changes, so marked a feature of the earlier stages, now cease, and the sphere shows only slight oscillating or trembling movements, due to the activity of the contained trypanosomes, moving restlessly within the comparatively tense envelope of the sphere, representing the periplast of the original trypanosome.

As the final moment approaches, the envelope of the sphere becomes more tense and rigid, its outline in optical section ever more nearly a perfect circle, until it bursts suddenly, setting free the contained trypanosomes. If the sphere bursts in its normal situation, that is to say within the cytoplasm of the host-cell, the liberated trypanosomes are seen moving actively for some time in the cell, from which

they escape one by one, passing out into the lumen of the stomach. But in the examination of stomachs freshly teased up, it is common to find spheres set free by the rupture of their host-cells. The spheres can then be seen to burst with such suddenness that they appear to explode; the impression given by the eye disappoints the ear, which misses the almost-expected report of the explosion; the trypanosomes set free scatter in all directions.

In one case a single cell was observed to contain three spheres, which were seen to burst successively; first one, then another, and then the third became resolved into free trypanosomes until the cell was full of active trypanosomes which appeared to be wriggling in a clear fluid. The cell retained at first its contours, but was apparently reduced to a spherical envelope, with no nucleus visible. Soon after the last sphere had become resolved the trypanosomes were seen to be escaping from a small area of the cell-wall, which then collapsed slightly; all the trypanosomes eventually found their way out at this part of the cell and swam off. In another case we timed our observations of a sphere and recorded the events in chronological order. It is not necessary to repeat the data already published, but they may be summarized as follows: A full-grown sphere was seen within a cell, moving and rotating by means of its flagellum, which was distinctly visible, and also exhibiting active metabolic form-changes; twenty-three minutes after it was first seen both the rotatory and the metabolic movements became much slower, and one minute later they ceased altogether, the alterations in the contour being slight, and due apparently to the very active movements of the contained trypanosomes; the flagellum had disappeared completely from view; the contour of the sphere then became very tense and rigid, and it burst after having been watched for twenty-eight and half minutes, or about five minutes after the disappearance from view of the flagellum and the cessation of the metabolic movements.

The trypanosomes set free are the long free stomach-type

already described, characterised by their great length, their stiff, crithidiomorphie form, and the more or less pronounced tendency to approximation of their two nuclei. The number produced in a sphere seems to vary considerably in different cases. We have attempted to count those which we have actually seen liberated within cells by bursting of spheres, and came to the conclusion that about eight was the usual number of trypanosomes produced. It is, however, very difficult to count accurately a number of trypanosomes which are at different levels in the cell, and, therefore, not all in focus at the same moment, and which at the same time are moving actively and changing their position. Moreover, the full-grown, tense spheres, which can be observed to burst, differ so greatly in size that there can be no doubt that the contained trypanosomes must vary considerably in number, and that if the average capacity of a sphere be eight trypanosomes, some spheres must liberate not more than four, others, perhaps twelve or sixteen, when they burst. These conclusions are confirmed by a study of fixed preparations; in the large spheres we find 8 (Pl. 37, fig. 86), 10 (Pl. 37, fig. 96), 13 (Pl. 36, fig. 41), and 14 or 15 (Pl. 37, fig. 94, and Pl. 36, fig. 42) *nn* and *NN*. From the results of counting the nuclei in a number of preparations, the most frequent number of *nn* in the largest spheres would appear to be 10 *nn*; the number of *NN* cannot always be made out, but where they can be seen their numbers are equal to, or slightly less than, those of the *nn*, indicating that the *nn* divide a little in advance of the *NN*. On the other hand, some spheres with but few nuclei appear to be nearly mature, having daughter-flagella so long that the nuclear division must have become much retarded, or perhaps ended altogether (Pl. 36, fig. 40, and Pl. 37, fig. 95). The permanent preparations confirm, therefore, the conclusion that while the number of trypanosomes produced is commonly eight or ten, it may be less, or as many as fourteen or more. The intracellular growth and multiplication of the trypanosome must depend on an interaction between the host-cell and

parasite, each a variable factor, and it is, therefore, not surprising if the result varies in different cases. Moreover, when a cell contains several parasites in different stages of multiplication, the cell may become exhausted before the younger individuals have time to grow to their full size.

The length of time required for a complete generation of intracellular multiplication is a point we have been unable to determine, as it is impossible to keep the living object alive under observation long enough. Moreover, the living trypanosomes in teased-up stomachs soon become abnormal, however carefully sealed up between slide and cover-slip, so that no data derived from direct observation of this kind could be entirely reliable. Until physicists and opticians have invented some method whereby the epithelial cells of the flea's stomach can be focussed under high magnification without dissecting or injuring the flea, it will not be possible to give a direct answer to such questions. Our observations cited above show that barely five minutes are required for a sphere to burst after the multiplication is complete, but that gives no clue to the length of time required for the growth and multiplication of the original trypanosome after it has penetrated the cell. From the fact, already noted, that the earliest intracellular stages were found by us about eight hours, and the earliest trypanosomes of the long stomach-type about twelve hours, after the flea had fed on an infected rat, it might be inferred that an intracellular generation required about four hours, but this computation cannot claim to possess any exactness.

It is also not possible to state how many successive generations of multiplication take place in the flea's stomach. Nöller states that at least two such generations, probably more, succeed each other. From the fact (to be described in more detail below) that the stomach-phase may be ended in some cases in eighteen hours, and in other cases may persist for four or even five days in unfed fleas (see above), it is evident that the number of generations of intracellular multiplication in the stomach must be a highly variable quantity.

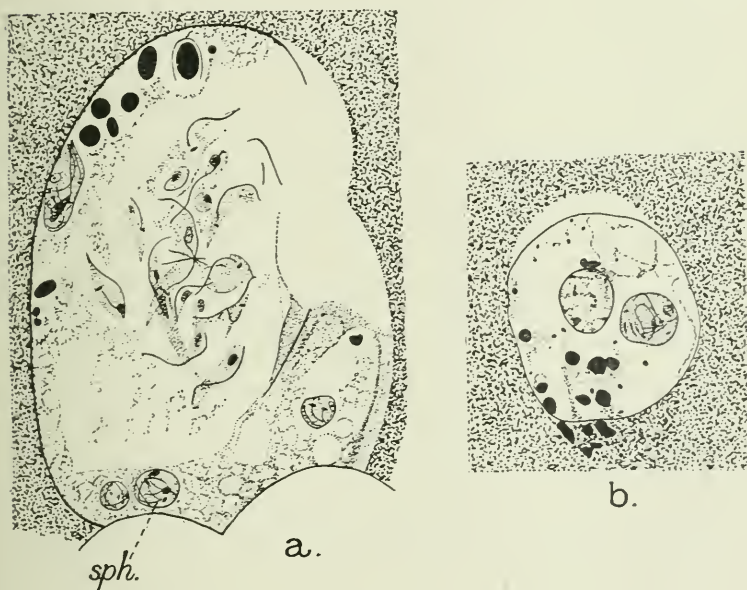
The foregoing account of the stomach-phase has been based almost entirely upon the study of the trypanosomes in the living condition and in smears. It was, in fact, all written, except for a few subsequent additions, before we had studied any sections. It remains now to supplement the description given above with the additional data furnished by the study of sections of the stomach, whereby a much more exact idea can be obtained of the relations of the trypanosome to the epithelium of the stomach.

When sections through a well-infected stomach are examined the trypanosomes are found usually to be both intracellular and extracellular in occurrence; that is to say, both free in the lumen of the stomach and contained within the cells of the epithelium. The extracellular trypanosomes occupy two different positions, speaking generally: they may be scattered throughout the blood-débris, including its most axial region, or they may be found only along the periphery of the sections, close to the epithelium and very often attached to it by the tip of the flagellum.

There can be no doubt that the trypanosomes found scattered through the central part of the blood-débris are for the most part forms in process of migration towards the rectum, their stomach-phase having been completed, but not all of them are to be interpreted in this way, since in many cases they are seen to be in clumps connected together by the tips of their flagella, and are obviously degenerative forms. In many cases also an infected epithelial cell is thrown off into the lumen, and may be seen there, crammed with trypanosomes, the product of the intracellular multiplication, which break out of the exhausted cell, and may possibly find their way into other epithelial cells again. The cell itself may become reduced to a mere sac containing fluid, in which a huge number of trypanosomes are squirming over one another (Text-fig. 3). If any of the protoplasm of the cell still survives it may contain a few spheres and other multiplication-stages. But that the forms free in the débris are for the most part migratory forms is indicated

by the fact that they occur with greater frequency towards the pyloric end of the stomach and sometimes only in this region, which may swarm with them, and in such cases they are always to be found in the intestine also.

TEXT-FIG. 3.



Exhausted cells containing intracellular stages of the trypanosome, from sections through the stomach of a flea thirty-six hours after the infective feed, $\times \frac{4000}{3}$. (a) A cell in process of being pushed off from the epithelium; the indented outline of the lower edge shows where it was in contact with epithelial cells in situ. The cell is reduced for the most part to a sac of fluid, in which trypanosomes are swimming, but round the edge some of the cytoplasm still remains, and in it are seen a few multiplication-stages (sph.), and also a few coarse black grains of fatty nature. The nucleus of the cell comes in another section. (b) A cell completely detached from the epithelium, the cytoplasm reduced to a few irregular strands and containing: (1) the nucleus at the centre; (2) a sphere to the right of it; (3) some coarse fat-grains, some of which have been extruded into the blood-débris.

On the other hand the trypanosomes that occur in close proximity to the epithelium are for the most part forms about to attack it, as shown by the fact that many of them are actually attached to the cells, and also by the frequency, it might almost be said the predominance, of the recurved form in this situation; at any rate, most, though by no means all, of those that are actually attached to the cells are recurved (Pl. 38, figs. 104, 105; Pl. 39, figs. 120-124). A certain number of recurved forms are also found, however, quite free from the epithelium, though close to it (Pl. 39, figs. 118, 119). The question at once suggests itself whether that represents the state of things as it was in life. It is impossible to extract a stomach from a flea without doing a certain amount of violence to it; and it is quite conceivable that the stresses and strains to which the wall of the stomach is subjected in pulling it out of the flea's carcase might well cause trypanosomes, previously attached, to become detached from the cells. We can but take the evidence as it is presented to us in our preparations, however, where we find that some of the attached trypanosomes are recurved and some not, and that some of the recurved forms are quite free from, though always close to, the epithelium. It would follow from these observations that in some cases the trypanosome assumes the recurved form after, in other cases before, attaching itself to the epithelial cell.

The attachment of the trypanosomes to the cells is peculiar; they are seldom attached to the extreme apex of the epithelial cell, but usually to its side (Pl. 39, figs. 121-124). The long flagellum runs down in the interspaces between two adjacent cells, and its extremity is often seen to be adherent to one of the cells close to the isthmus connecting it to its neighbour; the body of the trypanosomes usually projects into the lumen of the stomach above the general level of the epithelium, but sometimes the whole body is deep in between the cells. Sometimes two or three trypanosomes are attached side by side in such a situation. These statements apply to the usual columnar form of the epithelium with deep spaces inter-

vening between the cells; when the cell is flattened the trypanosomes may be attached at any point, and in the case of the young cells, which have but recently been produced from the epithelial buds and in which each cell is in contact with its neighbours along its side, without interspaces, the trypanosomes are attached to the convex apical region of the cell (Pl. 38, fig. 104), which may even contain early intracellular stages (Pl. 39, fig. 131).

As stated above, we have never been able to observe in the living state the actual penetration of the epithelial cell by the trypanosome; and in our sections we have searched in vain for appearances which could be interpreted as trypanosomes caught in the act of penetration. We have not found any trypanosomes half in and half out of the cell, from which it may be inferred, probably, that the actual penetration is effected very rapidly. But attention must be drawn here to a condition which is probably of significance in connection with this point. Trypanosomes of recurved type are found with the tip of the flagellum attached close to the base of the cell and with the rest of the body so closely applied to the side of the cell that careful focussing is necessary to determine that the trypanosome is still bodily outside the border of the cell and has not invaded it. Such appearances (Pl. 39, figs. 122, 123), suggests powerfully that the parasite, after attaching itself by the flagellum, forces its body into the cell. But the imagination must not be given too loose a rein when interpreting in terms of living activity the appearances seen in the dead fixed preparations.

The next stage is a recurved trypanosome within the cell (Pl. 38, fig. 107). Trypanosomes of the ordinary form (i. e. not recurved) are often seen in cells, but it is doubtful whether these represent individuals that have but recently penetrated the cell; if so, they might be forms which, after having been recurved, have temporarily straightened themselves out again, but it is on the whole more probable that they are trypanosomes which have been produced by the disruption of a sphere and which are about to leave the cell. In

support of the latter conclusion is the fact that we have seen trypanosomes of ordinary type only in cells that appeared more or less exhausted and liquefied, and especially often in detached cells. But, even if such forms represent recent arrivals in the cell, it is certain that they soon take on the recurved form. Consequently the recurved form may be taken in any case as the starting point of the intracellular development.

The growth of the recurved form into the sphere, the multiplication of its nuclei and flagella, and its ultimate fission to produce a bunch of trypanosomes, have been described in detail above, and only a few points in the development of the intracellular phase need be noted here. In the first place, in all preparations satisfactorily stained, it is seen clearly that flagella are present throughout, and that non-flagellated or leishmanial forms do not occur. This is so in all preparations stained with iron-hæmatoxylin and not over-extracted. In preparations stained by Giemsa's method the flagella are sometimes not to be seen, but in successful preparations they are exceedingly clear and sharp; it is always, however, one thing or the other, that is to say, we never find in one and the same preparation some parasites showing flagella and others none; either the flagella are invisible throughout the preparation, or are to be seen in every trypanosome, whether inside or outside the cells.

In sections the parasites are naturally very often in a mutilated condition, especially the large spheres, which may go through two or more sections. Consequently, it is not possible to be quite certain always whether a given sphere is tailed or not. But if the tail happens to lie in the plane of the section it may be shown very clearly, and then it can be seen that in some cases the tail is simply the original flagellum of the parent-trypanosome, projecting freely, while in other cases the tail includes the original flagellum with a prolongation of the trypanosome-body into which daughter-flagella may grow out, alongside of the parent-flagellum (Pl. 38, figs. 108, 110). In some cases, especially when the

host-cell is degenerating and breaking up, the flagellum of a sphere may project out from the cell (Pl. 39, fig. 132).

In concluding the description of the intracellular multiplication, we note that Chatton and Leger (1912), have described what they term a process of "agglutination and cytolysis" in *Leptomonas drosophilæ*, and suggest that the intracellular multiplication seen by us in the case of *Trypanosoma lewisi* in the stomach of the flea may be a phenomenon of the same order as that observed by them in *Leptomonas drosophilæ*. We cannot accept for a moment the suggestion that the behaviour of *T. lewisi* in the flea's stomach is anything but a quite normal process of multiplication. It is not incumbent upon us to criticise the statements of the authors, otherwise we might be tempted to inquire why they should think it necessary to interpret what they have seen in their *Leptomonas* as agglutination and cytolysis rather than as multiplication; but however legitimate such an interpretation may be for *L. drosophilæ*, we cannot admit it for the similar phenomena observed by us in *T. lewisi*.

APPENDICES TO THE DESCRIPTION OF THE STOMACH-PHASE.

Having given in the foregoing pages an account of the intracellular multiplication of the trypanosomes in the stomach, it remains to discuss the relations of parasite to host and vice versa. Under this theme are comprised (1) the frequency of occurrence of the intracellular multiplication in fleas exposed to infection, (2) the type of epithelial cell attacked by the parasite, (3) the relation of the intracellular stages to the infected cell, (4) the effects of the parasites on the cells, and (5) the relation of the infection as a whole to the stomach. The first of these problems is a statistical one, and is best studied in fresh or preserved film-preparations; the remaining four questions are best studied in serial questions.

(1) The Occurrence of the Intracellular Multiplication.

The following is a summary of our observations on the occurrence of the intracellular multiplication from twelve hours onwards, not counting those fleas that were used for sections:

At 12 hours approximately, after the fleas had fed on an infected

rat, intracellular multiplication was found in one flea out of ten examined :

At 15 hours, in	1 flea out of	15.
At 18	2 fleas	15.
At 20	1 flea	2.
At 24	40 fleas	240.
At 36	2	5.
At 48	5	20.
At 60	none	22.

At three, four and five days after infection, we only found trypanosomes in the stomachs of fleas that had been kept isolated and starved ; in those that had been allowed to feed again on a clean rat no trypanosomes of any kind, free or intracellular, were found in the stomachs. Counting, therefore, only starved fleas, we found intracellular stages :

At 3 days, in	5 fleas out of	16.
At 3½	1 flea	7.
At 4	2 fleas	13.

Later than five days we have never found them.¹

The total of all these observations is that we have found intracellular stages of multiplication in the stomachs of sixty out of 365 fleas examined from twelve hours to four days after infection, or approximately 16·5 per cent. There is no doubt, however, that this figure falls below the actual percentage of fleas in which the trypanosomes succeed in establishing themselves in the stomach and passing through their intracellular phase when ingested by the flea, for at least two reasons. In the first place, many of the 240 fleas dissected at twenty-four hours after the infective feed were examined very hurriedly and cursorily, the object being to find fleas in which the intracellular stages were swarming, for the purpose of making permanent preparations of these stages, and when they were not found quickly in the teased-up stomachs examined in the fresh state, the search was abandoned ; consequently in many cases in which the intracellular stages were scanty they may well have been overlooked, and would probably have been found after more prolonged search.² In the second place, as regards the fleas examined

¹ Since this page was written we have found intracellular stages in a flea which had been fed on an infected rat five days previously, and since then had been kept starved.

² Compare especially the figures given below (Text-figs. 4-12) of stomachs reconstructed from serial sections ; it will be seen that there may be instances in which only one or two small patches of epithelium are infected, and in such cases it would be a lucky accident if the infection were discovered in looking at the freshly teased-up stomachs, even after a prolonged search.

at later periods than twenty-four hours, it is highly probable that in many of these the intracellular multiplication may have been completed and this stage passed. In one of our series of sections of a stomach preserved thirty-six hours after infection the stomach itself contained no trypanosomes of any kind, but two clumps of typical crithidial forms were found attached in the intestine behind the pylorus (Pl. 42, fig. 273); in this case the stomach-phase must have been over, assuming that it is a necessary and essential part of the developmental cycle. In Table M it will be seen that in several cases fleas dissected at two or two and half days after the infective feed had no trypanosomes in the stomach but developmental crithidial forms in the rectum. The absence of intracellular multiplicative forms in the stomach of a flea examined more than twenty-four hours after the infective feed is, therefore, no proof whatever that this phase has not been passed through in that flea.

When due weight is given to both these considerations, namely the probability that scanty infections may be overlooked in hasty examinations of stomachs in the fresh conditions, and the fact that the stomach-phase may be passed through rapidly and ended very soon, there can be no doubt that the true percentage of fleas in which it must take place must be considerably higher than the figure, 16.5, derived from actual observation. We should probably not be far wrong in putting it above 20, a result agreeing fairly closely with the percentage of fleas found by direct experiment to become infective after having fed on an infected rat (see below, p. 663), and thus supporting our belief that the intracellular multiplication is necessary for the trypanosome to establish itself in the flea, and is an indispensable part of the life-cycle; a belief which is not, however, under the circumstances, capable of direct proof or verification.

It is at least evident from the figures given that the investigator must not expect to find the intracellular multiplication in more than a fraction ($1/6$ or $1/5$) of the number of fleas examined which have fed but once on an infected rat; and that he is most likely to find them from eighteen to thirty-six hours after the trypanosomes have been ingested by the flea.

(2) The Type of Cell attacked by the Trypanosome.

With regard to this point we have to note, in the first place, that we have never seen intracellular stages of the trypanosomes in the cells of the epithelial crypts. In this we are in agreement with Nöller (1912), who has also studied sections of the flea's stomach. Extracellular trypanosomes are sometimes seen attached to the exterior of cells which are passing out from the crypt, but never inside any cell which is not definitely a part of the general epithelium. It is possible that

the separation which, as described above (p. 494), takes place between the epithelial cells until they are connected only by the isthmus at the base, makes it easier for the trypanosomes to penetrate into them, since the appearances seen in sections suggest that the attack is usually made on the side of the cell (Pl. 39, figs. 120-124); the occasional, though rare occurrence, however, of intracellular stages in quite young cells (Pl. 39, fig. 131), shows that the trypanosomes can penetrate into epithelial cells before the separation between them has developed.

The degenerated epithelium, full of fatty deposits which blacken after treatment with osmic mixtures, is also not attacked by the trypanosomes. It is true that intracellular stages may sometimes be found in a degenerated cell, but in such cases the cell has been thrown off from the epithelium and the parasites are in the condition of large spheres ripe for breaking up into trypanosomes (Pl. 39, fig. 135); or of masses and clumps of trypanosomes that have evidently been liberated by the recent disruption of a sphere (Pl. 38, fig. 114). It may be supposed that in these cases the cell was attacked by the trypanosomes before degeneration had set in; the process of degeneration may have been hastened by the action of the parasites.

The trypanosomes attack by preference the fully-formed, but still young and vigorous cells, which may contain granules of the normal type and even yellow bodies, but no fatty deposits; cells which may be well characterised as adolescent in type, and which stain a clear, light-grey with iron-haematoxylin after Flemming-fixation. It is in such cells that the earlier stages of the intracellular multiplication are to be found in a flourishing condition and often in considerable numbers; but if the trypanosomes are numerous the cell soon becomes exhausted.

(3) The Relation of the Trypanosomes to the Cells.

The intracellular parasites are found most frequently in the apical expanded region of the cell, where the cytoplasm is usually of a loose spongy texture (Pl. 39, fig. 126). Sometimes, however, the trypanosomes are seen below the nucleus and occasionally there may be parasites lodged above and below the nucleus in the same cell (Pl. 39, fig. 134). Since the position of the nucleus in the cell is subject, as has been pointed out above, to variation, no special significance attaches to this point. It may be noted, however, that when a sphere is situated, as regards the principal mass of its body, above the nucleus, the tail of the sphere may run down to the base of the cell (Pl. 38, fig. 108).

In epithelium of the flattened form the intracellular parasites are lodged beside the nucleus, which is often pushed to one side of the cell.

(4) The Effects of the Trypanosomes on the Epithelial Cells.

In describing the pathological effects of the trypanosomes, we may begin with the cases where a cell is attacked by a few, not more than two or three, parasites, and has been but little affected by them (Pl. 39, figs. 126, 127). It is seen that the parasite is lodged in a distinct space or vacuole in the cytoplasm, a vacuole which is not to be interpreted as an artefact due to shrinkage from the action of reagents, but as produced by the liquefactive or absorptive action of the parasite on the cytoplasm, since the vacuoles containing the intracellular parasites can be seen very clearly in the living cell. Similar effects produced by *Toxoplasma gondii* in the peritoneal cells of the mouse have been described by Miss Pixell.¹ In the living condition, as stated above, the trypanosomes move freely by means of their flagella in the liquefied cytoplasm, and when there are many parasites in the same cell they can be seen to jostle one another and even to bump against the cell-nucleus. Owing to the liquefaction of its cytoplasm the cell becomes empty and exhausted, and in its final stage is reduced to the condition of a sack, containing fluid, which may be crammed with trypanosomes produced by the process of multiplication within the cell; remnants of the cytoplasm may persist on the wall of the sac, and the cell-nucleus is also to be found at some point, generally adherent to the wall. Often these exhausted cells contain fatty deposits and also the brownish-yellow bodies described above.

The effect produced on the parasitised cell as a whole is a more or less distinct hypertrophy, more obvious when the contained parasites are present in large numbers (Pl. 38, fig. 114). Then the cell often becomes very much enlarged, but this enlargement appears to be brought about in many cases not solely by the hypertrophy of the individual epithelial cell, but also by the fusion of distinct cells: at least this is the only way in which we are able to interpret the large multinucleate cells, containing numerous parasites in all stages of their multiplication, that are frequently to be found. Mitoses, or any other forms of nuclear multiplication are never seen in the epithelium, and the very large size of these multinucleate cells (Pl. 39, fig. 128; compare Pl. 44, fig. 313), certainly indicates cell-fusion having taken place. We have also seen multinucleate epithelial cells not containing parasites: but such cases might very well be those in which the intracellular multiplication was ended and the daughter-trypanosomes produced had escaped from the cell.

As the cells are exhausted and destroyed by the parasites there is a pronounced tendency for them to be thrown off from the epithelium.

¹ 'Proc. Roy. Soc.,' (b), lxxxvii, p. 73. (Pl. ix, figs. 3-6).

In the sections infected patches of epithelium are of frequent occurrence, in which the infected cells are seen bulging far out from the general level of the epithelium into the lumen of the stomach, giving sometimes an appearance like a bunch of grapes (Pl. 44, figs. 313 and 315). In such protuberances every cell, as a rule, contains numerous parasites in all possible stages of multiplication, in progress or completed. In one of our series there is a stomach in which no parasites are to be found within the epithelial cells *in situ* in any part of the stomach-wall, but in the fore part of the stomach there are very numerous intracellular parasites, all contained in cells completely detached from the epithelium. On the other hand very large patches of epithelium may be found infected without the cell being thrown off; in one of our series there is a stomach, which, in one part shows infected cells the whole way round the section, except in the epithelial crypts (Text-fig. 9), but there is very little detachment of epithelial cells. It is evident that the expulsion of the infected cells from the epithelium is a measure partly of the intensity of the infection, i. e. the number of parasites contained in each cell, and partly of the length of time during which the parasites have been acting on the cells; it is not till the cells are becoming exhausted and incapable that they are ejected from the epithelium.

From a study of the extracellular trypanosomes, which occur in close proximity to the epithelium, and more especially those actually attached to it, it is evident that the extent of the epithelial areas simultaneously attacked may vary very greatly in different cases. Sometimes only an attached trypanosome is to be seen here and there, scarcely so much as one in each consecutive section on the average; this corresponds to the frequent occurrence of solitary epithelial cells, or very small groups of them, containing parasites. Sometimes, on the contrary, all the epithelium on one side of many consecutive sections will be seen to have trypanosomes adherent to it, while on the other side of the same sections not a trypanosome is to be seen (compare Text-figs. 4-12). What determines the extent and distribution of the attacks it is impossible to say, but at least one necessity is probably the presence of regenerated "adolescent" epithelium.

It should be mentioned finally that in one of our series there is a stomach which in its hinder region is almost entirely denuded of epithelium. Vast numbers of trypanosomes are seen in all parts of the stomach-lumen, but swarming most thickly near the wall, on which only the epithelial crypts remain intact. Remnants of the general epithelium occur here and there in the form of broken-down cells containing the final stages of the intracellular multiplication, but for the most part the epithelium is gone altogether. In the anterior region of this stomach, trypanosomes are less abundant and the epithelium is in process of regeneration. It is surely a fortunate circumstance for the

insect host that its epithelial crypts of regeneration are immune to the attacks of the trypanosomes; were it not so, it is hardly credible that the flea could survive the extensive destruction of the epithelium that may occur with an intense infection.

(5) The Relation of the Trypanosome-Infection, as a Whole, to the Stomach of the Flea.

This point can best be made clear by describing a few examples, which will give a more graphic picture of the variations seen in different fleas than can be obtained from a general description. It must be stated, in the first place, that, as already pointed out above, in stomachs of fleas preserved eighteen, twenty-four, or thirty-six hours after having been fed on a well-infected rat, the trypanosomes may have entirely disappeared, having been digested with the blood and failed to establish themselves. Consequently, in many of our series of sections, some or all of the stomachs contain no trypanosomes at all. On account of the great expenditure of labour and time involved in searching through a complete series of sections of a stomach we have not attempted to compile any statistics of the numerical proportion of infected to non-infected stomachs, as we have done in the case of stomachs teased up and examined fresh, the latter being a method much less laborious, though not so exact, for determining whether a stomach contains trypanosomes or not.

In the examples we are about to give we deal only with those stomachs in which trypanosomes have been found, and in order to exhibit at a glance the state of things in each stomach we have made reconstructions of a certain number of stomachs in the following manner:

(a) To show the distribution of the cells containing intracellular stages, the stomach is imagined as cut open from the intestine to the proventriculus along a line which corresponds to the southernmost (lowest) point in the transverse sections, but which is a purely arbitrary line so far as the flea is concerned and does not correspond to any definite anatomical plane of the insect. The wall of the stomach, supposed to have been cut open along this line, is further imagined as laid out flat. Of each stomach reconstructed in this way a diagrammatic sketch was made on paper ruled in millimeter squares to a definite scale, namely, 1 mm. to each transverse section of $6\ \mu$ in thickness, and in the sketch three meridians are put in with dotted lines, the middle one to represent the northernmost (uppermost) point of the sections, while the meridians to the left and right represent respectively the extreme western (left-hand) and eastern (right-hand) points in the section. After making a diagrammatic sketch in this manner the series of sections was searched through and all infected cells were mapped out

in the reconstructions, being represented by little circles placed in the line corresponding to the number of the section, and in the relation to the meridians that indicate its position in the section. For instance, if an infected cell is found in the twenty-seventh section, to the north-east of the section, the circle representing it is put into the diagram 27 mm. from the anterior end of the stomach and midway between the meridians N. and E.

In this way a graphic representation, accurate to scale in the longitudinal direction, is obtained of the distribution of the infected cells in the epithelium. It is important to remember that each little circle represents not merely a single intracellular stage of the trypanosome, but an infected cell which may contain many such stages.

(b) To show the distribution of the extracellular trypanosomes the diagram is supposed to represent the unopened stomach, and the trypanosomes that are in close proximity, or attached, to the epithelium are put in along the sides, while those scattered free in the *débris* are represented dispersed through the diagram, in the position corresponding to the number of the section in which they occur; when very numerous, however, it is impossible to represent them accurately, and they are merely crowded in as thickly as possible in the diagram.

It is very important that the reader should understand clearly that in each of our diagrammatic reconstructions of the stomachs two distinct conventions are combined, as it were superposed one on the other. Originally our intention was to have given two diagrams for each stomach, one reconstructed according to method (a) to show the distribution of the intracellular parasites, the other according to method (b), indicating the occurrence of the extracellular trypanosomes. To have given two diagrams for each stomach, however, would not only have taken up much space, but would not have shown the state of affairs so graphically as when the two diagrams are combined into one and no confusion can arise if it is clearly understood that two different methods of reconstruction are combined in each figure. The fact that the attached trypanosomes are represented adherent to the sides of the diagram, for example, does not mean that they are attached only along the southern meridian; they are attached at any point in the section, but had they been indicated in the diagram in the exact meridian in which they occur the eye would not have distinguished them from the free forms.¹ To indicate the exact position of the attached trypano-

¹ It was suggested by a friend that the attached trypanosomes might have been distinguished from those that are free by drawing at one end of them a little transverse line or semicircle, to indicate the attachment to the cell; but where the trypanosomes are thickly crowded, as in Text-fig. 10, it would have been impossible to distinguish clearly the free and the attached forms in this way.

somes it would have been necessary to have shown the attached and the free trypanosomes in separate diagrams; either to have put in the attached forms in the diagram showing the infected cells, according to method (a), or to have constructed three diagrams showing the infected cells, the attached trypanosomes, and the free trypanosomes on each diagram separately.

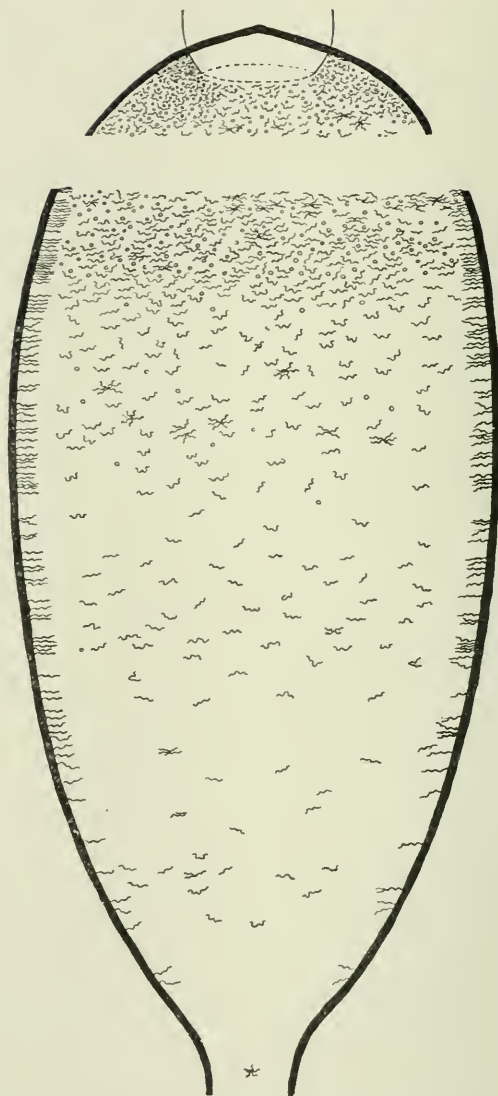
The reconstructions were made, as has been stated, on a definite scale as regards the length of the stomach, namely, on the scale of 1 mm. to 6μ , that is to say at a magnification of $\frac{1000}{6}$, or 167 approximately. In the reproduction, however, the diagrams have been reduced to two-thirds of their original size, and appear, therefore, at a magnification of $\frac{2000}{18}$, or 111 approximately, with the exception of figs. 3 and 11, which it has been necessary to reduce to one-half on account of the very large size of the stomachs, and which, therefore, appear at a magnification of about 83.5, while fig. 9 has been reduced to one-third. The diagrams show the great variations in the size of the flea's stomach. The small stomachs are males, the large ones females. The number of sections in a series cut through a small stomach was generally about 125, in a large stomach from about 220 to 300. This corresponds in a remarkable way to some observations we had made and recorded long previously to cutting the sections—on the feeding of the fleas. We found that a male flea took about a minute and a quarter to fill its stomach, a female about two minutes and a quarter.

The stomachs reconstructed by us were all from fleas fed twenty-four or thirty-six hours before being dissected and preserved. We will begin with the fleas of thirty-six hours, because we possess a very good series of this period, which has been studied very carefully by us, and which, having been preserved in Flemming's fluid and stained with iron-haematoxylin, shows the state of the epithelium and the stomach-contents particularly well.

(a) Thirty-six hours after feeding.—A batch of nine stomachs, all preserved at the same time, stuck on the same piece of liver and cut in the same block of paraffin. Owing to an accident to the block the two stomachs near one end of the liver were damaged; the anterior half of one of these stomachs and a small part of the anterior end of the other were broken away. In what remained of these two stomachs no trypanosomes of any kind were found, and the same was the case with one of the other stomachs in this series. There remain six stomachs to be described.

(1) (Text-fig. 4). The stomach is a large one, going through about 260 sections, corresponding to a length of about 1.56 mm., exclusive of the proventriculus. The blood-débris is large in amount and stained

TEXT-FIG. 4.



Reconstruction of a stomach in the manner described in the text, to show the distribution of the intracellular and extracellular trypanosomes. The portion left blank is where some of the sections were accidentally destroyed. No meridians have been put in because, in this instance, all the intracellular trypanosomes were in detached cells. 36 hours. Magnified about 83.5.

yellow. The epithelium is for the most part flattened and very black, especially at the anterior end, where also there are very many detached cells. There are also a great many yellow necrosed cells, either in the form of extensive patches in the epithelium in situ, or of detached cells loose in the blood-débris.

Extracellular trypanosomes abundant, especially round the detached cells in the anterior region. In the posterior half of the stomach they are much fewer, and almost all peripheral in position or attached to the epithelium.

Intracellular trypanosomes very abundant in the anterior fourth, but only in detached cells, none in the epithelial cells in situ. Passing backwards they become scarcer, and in the posterior half of the stomach none are found.

Since the intracellular parasites in this stomach are found only in detached cells, the meridians have been omitted in the reconstruction, it being impossible to refer the infected cells to their proper position in the stomach-wall.

Interpretation.—This is a stomach in which the epithelium is in the final stages of degeneration, especially anteriorly, but regeneration has not begun. There has been an extensive intracellular infection in the most anterior region, represented now by advanced stages in the detached epithelial cells, and swarms of free trypanosomes are attaching themselves in preparation for a fresh attack. There are also clumps of degenerative forms free in the blood.

(2) The stomach goes through about 220 sections corresponding to a length of 1.32 mm. Blood-débris in moderate quantity, stained yellow; epithelium clear, granular, containing many grains and pseudospheres. No trypanosomes in any stage found in the stomach, but in the intestine, behind the pylorus, in a large clump of attached crithidial forms.

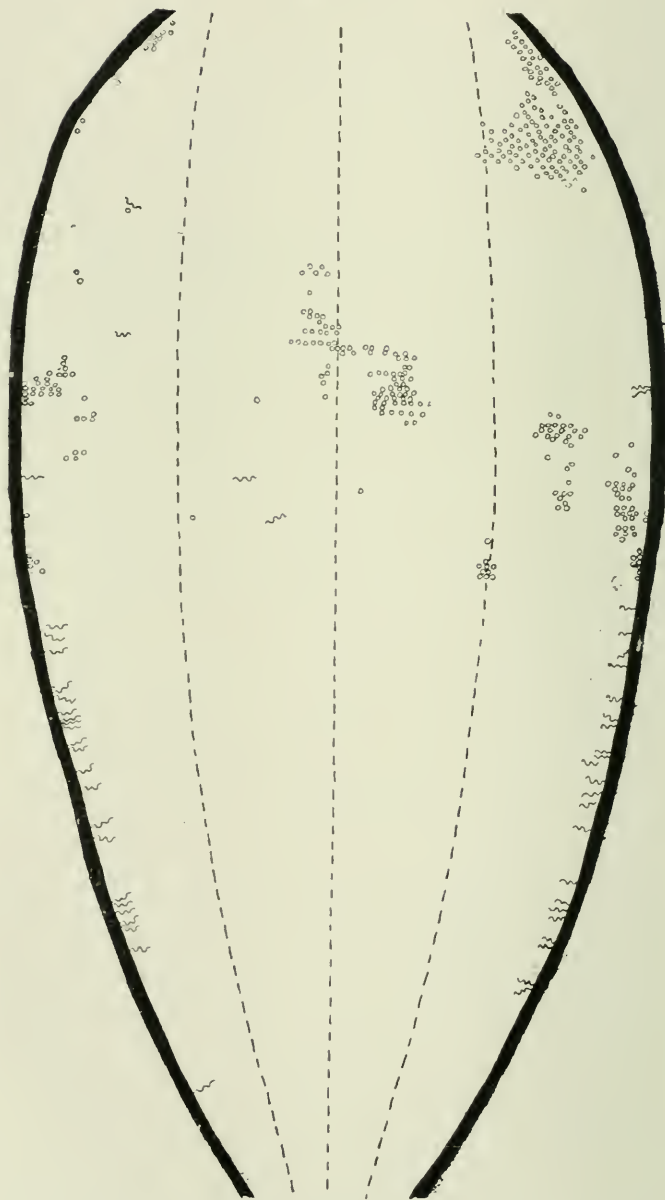
Interpretation.—A stomach which has recently been regenerated and in which the stomach-phase of the trypanosomes is completed and past.

(3) (Text-fig. 5). A large stomach going through about 260 sections. Blood-débris about half absorbed; stains grey. Epithelium for the most part clear, columnar, very granular, with coarse grains and pseudospheres and many yellow bodies. There are a fair number of detached cells, some of them showing yellow necrosis, scattered through the stomach; in the posterior region there are some black degenerated cells still in situ or in process of detachment.

Extracellular trypanosomes very scarce in the anterior half of the stomach; in the posterior half they are more numerous and all attached to the epithelium or peripheral in position.

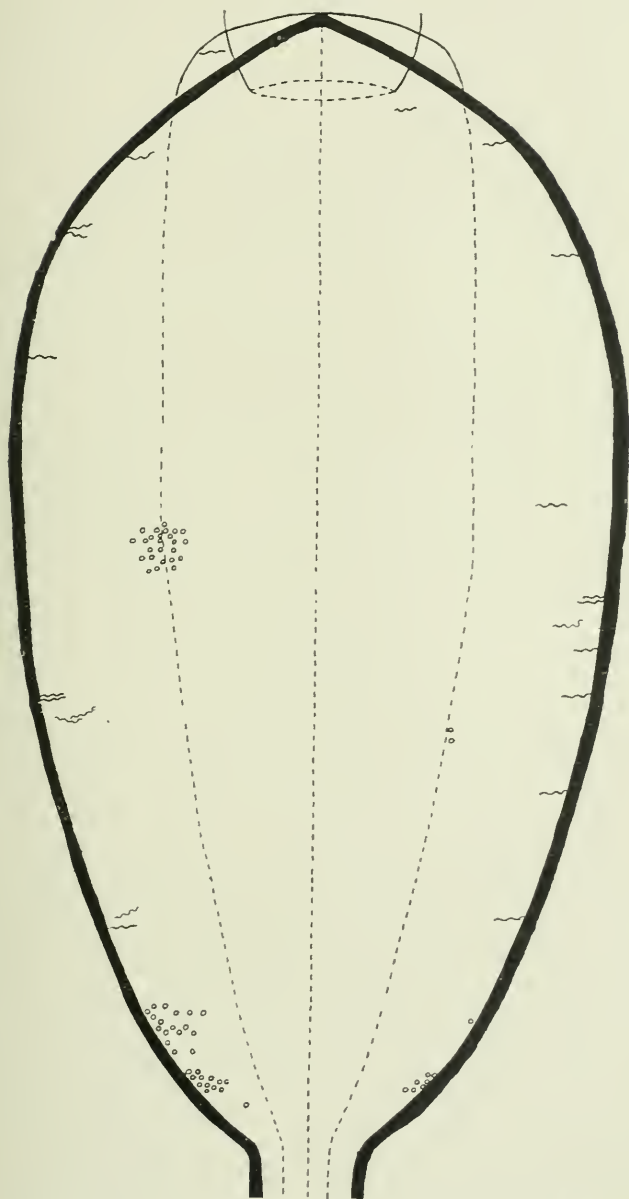
Intracellular trypanosomes are found only in the anterior half, where there are scattered patches, variable in extent, of infected epithelium.

TEXT-FIG. 5.



Reconstruction of another stomach. The series of sections began just behind the proventriculus. 36 hours. Magnified about 111.

TEXT-FIG. 6.



Reconstruction of another stomach. 36 hours. Magnified about 111.

Interpretation.—A stomach very recently regenerated, the process scarcely complete in the posterior region. In the anterior half practically all the trypanosomes have penetrated into the epithelium. In the posterior half an attack is beginning, but all the parasites are still extracellular.

(4) (Text-fig. 6). A large stomach going through 223 sections. Blood-débris as in last. Epithelium as in last, but less granular and with no yellow bodies; no black cells in situ, but a few detached.

Extracellular trypanosomes very few in number, scarcely more than twenty in the whole series; all attached to the epithelium or close to it.

Intracellular trypanosomes in two fairly extensive patches of infected epithelium, one about the middle of the stomach, one close to the pylorus. In one of these patches the cells are badly affected; in the other they are more normal.

Interpretation.—A feebly-infected stomach, recently regenerated, in which almost all the trypanosomes are intracellular.

(5) The stomach runs through 217 sections. Blood-débris large in amount; stained yellow. Epithelium: (a) in the first 130 sections mostly very dark, but interspersed with clearer patches of cells; many detached cells, some black, some yellow; (b) in the hinder $\frac{5}{8}$, approximately, of the stomach the epithelium is mostly clear, not very granular, with a few patches of black cells in situ, and many detached cells, black or yellow.

Extracellular trypanosomes all confined to the anterior region, in rather scanty numbers for the most part peripheral in position and often attached.

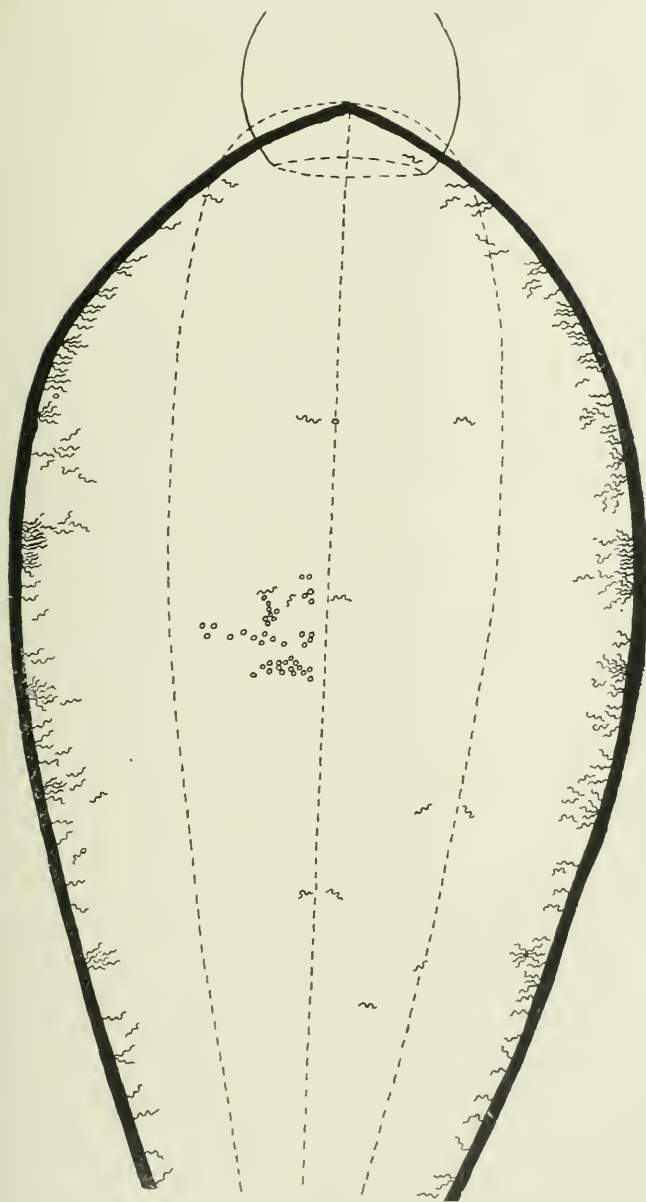
Intracellular trypanosomes found only in the posterior region; three patches of infected epithelium, one fairly large, running through forty-six sections, the cells considerably modified, and two small patches; also a detached cell containing a large sphere in section 132 (rather far forward).

Interpretation.—A stomach in which regeneration is just beginning in the anterior region and is fairly advanced posteriorly. In the anterior region the presence of necrosed cells indicates that there has been a recent attack, but at present there are only extracellular trypanosomes; in the posterior region there are fairly extensive patches of infected epithelium and no extra-cellular trypanosomes.

(6) (Text-fig. 7). The stomach runs through about 240 sections. Blood-débris large in amount; yellow. Epithelium clear, columnar, and with many granules, and in places yellow bodies; interspersed are a few black or yellow cells, singly or in patches, in situ, and detached cells are also fairly numerous.

Extracellular trypanosomes numerous; nearly all close to the epithelium and many attached along the whole length of the stomach.

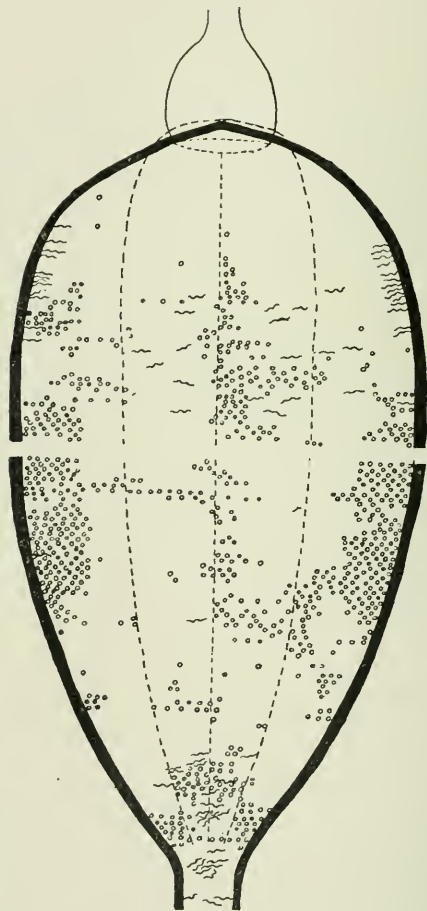
TEXT-FIG. 7.



Reconstruction of another stomach. Owing to the exigencies of space the hinder part of the stomach, in which there were no trypanosomes, has been cut off. 36 hours. Magnified about 111.

Intracellular trypanosomes practically confined to a fairly extensive patch of epithelium about the middle of the stomach.

TEXT-FIG. 8.



Reconstruction of another stomach. The gap in the middle of the figure shows where three sections were accidentally destroyed. 24 hours. Magnified 111.

Interpretation.—A stomach which has undergone regeneration, which is scarcely completed. A previous intracellular generation of trypanosomes, indicated by the presence of necrosed cells, has given rise

to numerous free trypanosomes that are attacking the regenerated epithelium, and have penetrated it and established themselves in the cells in a few places.

(b) Twenty-four hours after feeding.—(7) (Text-fig. 8). From a series preserved in Maier's fluid.¹ A small stomach, running through about 150 sections. In the anterior quarter, or thereabouts, of the stomach are a fair number of attached forms. Behind this region begins a very intense intracellular infection which diminishes somewhat in the posterior fourth, except for an extensive patch close to the pylorus. Through all this region there are practically no attached trypanosomes, but there are a few scattered through the *débris*, which become more numerous towards the pylorus, and are found passing down the intestine, where some of them are attached to the lining.

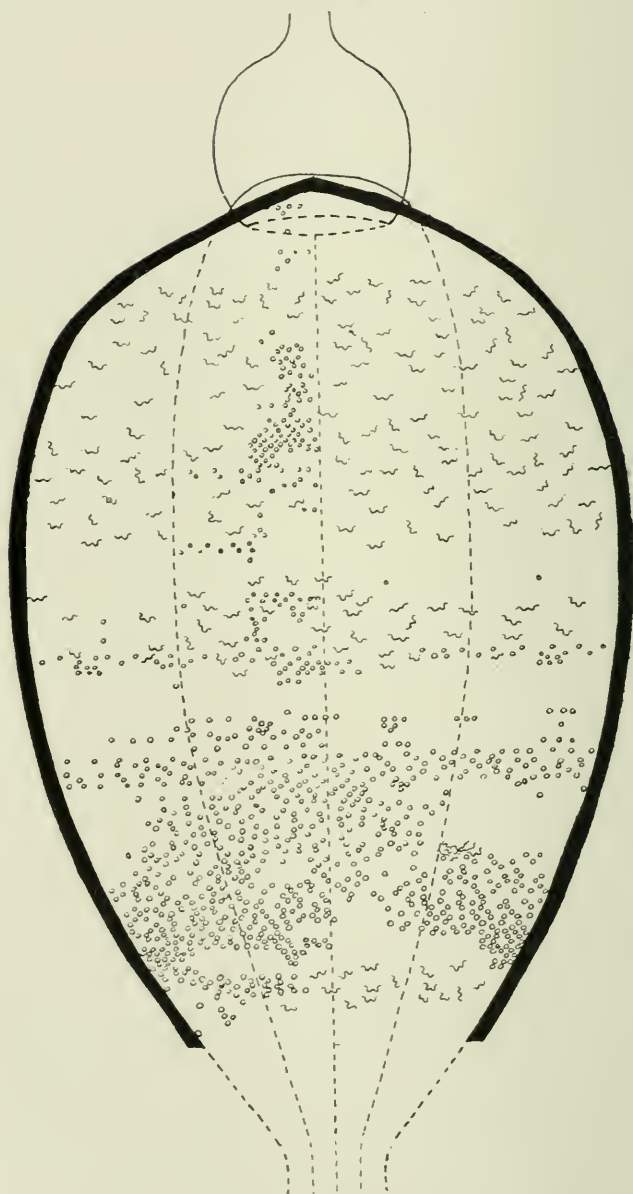
Interpretation.—The intracellular multiplication is at its height and the trypanosomes are beginning to pass on to the rectum, but at the extreme anterior end a fresh attack is developing.

(8) From the same series as the last. A small stomach running through about 154 sections. In the anterior twenty sections the epithelium is columnar, normal, or in places tending to degenerate, with no intracellular trypanosomes, and very few free trypanosomes in the sections. For about forty sections behind this there is an intense infection of the epithelium, which in places is largely destroyed or broken up, and there are great numbers of extracellular trypanosomes, some scattered through the *débris*, others in close proximity to the stomach-wall. In the next fifty sections, approximately, the epithelium is almost entirely destroyed; only the crypts remain, with here and there a few broken-down cells still adherent to the wall and containing late or completed stages of the multiplication; there are also in this region enormous numbers of extracellular trypanosomes, mostly in close proximity to the wall of the stomach, but also scattered through the *débris*, in which there are, in addition, masses of detached, broken-down cells, many of them containing large spheres or clumps of trypanosomes. In the twelve sections behind this region the number of trypanosomes and detached cells diminishes rapidly, and in the last thirty-two sections there are no trypanosomes, though still a few detached cells.

Interpretation.—A stomach with an extraordinarily intense infection, which, in the middle region, has destroyed the epithelium completely. The intracellular multiplication has produced vast numbers of trypanosomes, which are not beginning as yet to migrate backwards

¹ In the case of stomachs (7) to (15) the slides of the series were stained alternately with the iron-hæmatoxylin-Lichtgrün-combination and by Giemsa's method.

TEXT-FIG. 9.



Reconstruction of another stomach. The series of sections was not continued quite as far as the pylorus. 24 hours. Magnified about 111.

to the rectum, judging from their absence in the posterior region of the stomach.

(9) From the same series as the last. A large stomach running through about 234 sections. Epithelium almost everywhere columnar, a few flat cells: very few detached.

Extracellular trypanosomes fairly numerous towards the hinder end, mostly peripheral and many attached, a few scattered in the débris. No intracellular trypanosomes found.

Interpretation.—A stomach in which one or more generations of intracellular multiplication have been completed and a new attack on the cells is beginning.

(10) (Text-fig. 9). From the same series as the last. A large stomach, but unfortunately not quite enough sections were mounted and the series ends before the pylorus. Blood-débris large in amount everywhere.

In the anterior half of the stomach there are a few extracellular trypanosomes scattered through the blood-débris, very few attached: there are also some intracellular parasites, for the most part scanty, but in one place there is a fairly extensive patch of infected epithelium.

In the posterior half there is a very intense infection of the cells, which in many sections is seen all round the section, or interrupted only by the epithelial crypts. In this region, extracellular trypanosomes are practically absent; one clump, apparently of degenerative forms, was found, and in the hindermost region free trypanosomes, scattered in the débris, begin to appear abundant in the pyloric region.

Interpretation.—Over a large extent of the stomach the intense infection of the epithelium has absorbed, so to speak, all the free trypanosomes except the degenerating forms.

(11) From another series preserved in Maier's fluid. A stomach of moderate size, running through about 180 sections.

Extracellular trypanosomes swarming in the posterior region of the stomach and in the intestine; towards the anterior end they diminish in number, but are to be found right up to the proventriculus. Almost all these trypanosomes are free in the débris, very few are attached.

No intracellular trypanosomes found.

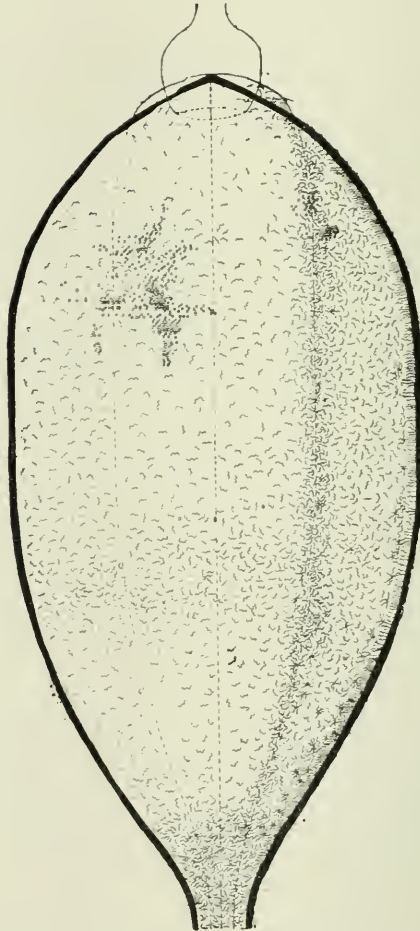
Interpretation.—A stomach in which the intracellular multiplication is practically ended and the trypanosomes, present in large numbers, are passing down to the rectum.

(12) Text-fig. 10). From the same series as the last. A very large stomach, running through about 320 sections, corresponding to a length of nearly 2 mm.

Extracellular trypanosomes occur in vast swarms along the whole

length of the stomach; most of them are peripheral in position occurring all along the east (right-hand) side of the sections, and many

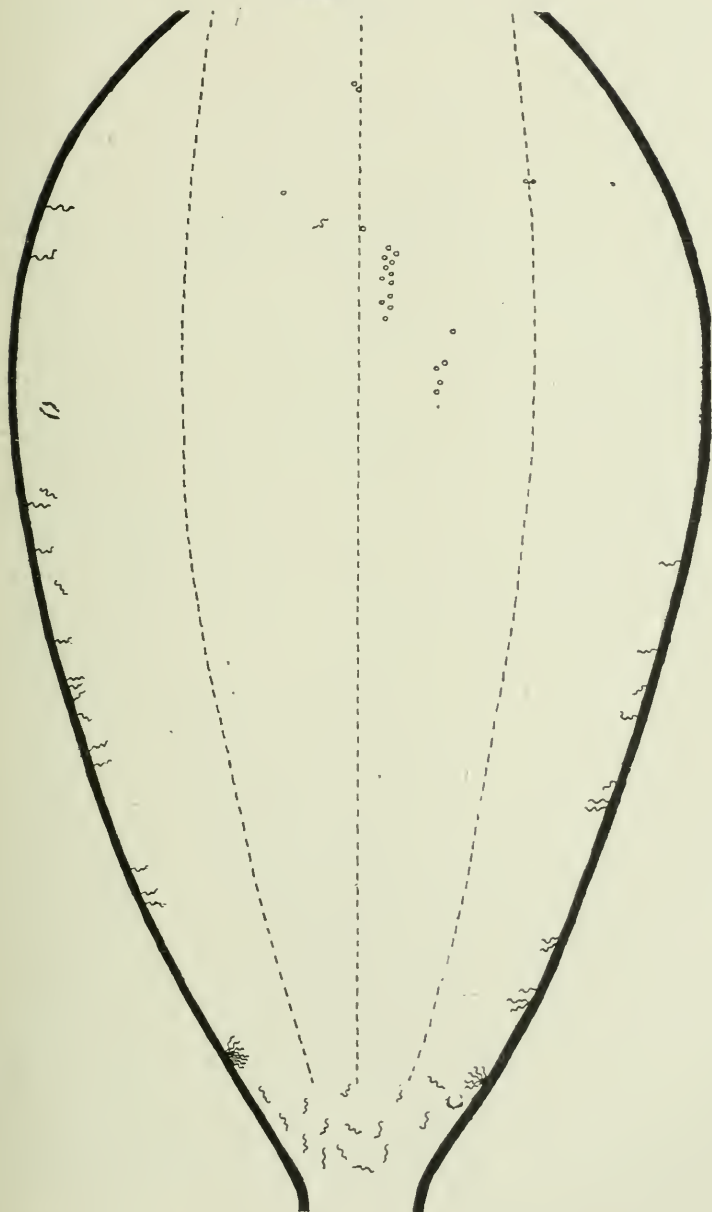
TEXT-FIG. 10.



Reconstruction of a very large stomach. 24 hours. Magnified about 55.5.

of them are attached. Trypanosomes also occur, however, scattered through the débris in all the sections, but more especially in the posterior half of the stomach, and swarms of them are seen in the

TEXT-FIG. 11.



Reconstruction of another stomach. The anterior part of the figure, from just behind the proventriculus, has been cut off owing to exigencies of space, but there were no trypanosomes in the part that has been cut off. 24 hours. Magnified 111.

pyloric region passing down the intestine. Many of these free forms, whether central or peripheral in position, are in clumps and are possibly degenerative.

Intracellular trypanosomes are not found in the posterior half of the stomach, but towards the middle a few detached infected cells are found here and there. In the anterior region there is a very large patch of infected epithelium in situ and also two smaller patches.

Interpretation.—Intracellular multiplication is proceeding actively in the anterior region, and a new attack on the epithelium is preparing all along one side of the stomach. At the same time, trypanosomes are passing down in large numbers towards the rectum and there are many degenerative clumps.

(13) (Text-fig. 11). From another batch preserved in Maier's fluid. A large stomach running through 255 sections.

Extracellular trypanosomes very scanty, practically confined to posterior half of stomach, all attached except in pyloric region, where there are a few scattered freely in the debris. Many of the attached trypanosomes are in clumps, perhaps degenerative.

Intracellular trypanosomes only in anterior region, very scarce.

Interpretation.—The stomach-phase seems to be maintaining itself with difficulty in this flea; the trypanosomes are few in number and largely degenerative, but a few are passing backwards.

(14) Text-fig. 12). From the same batch as the last. A large stomach, running through about 225 sections.

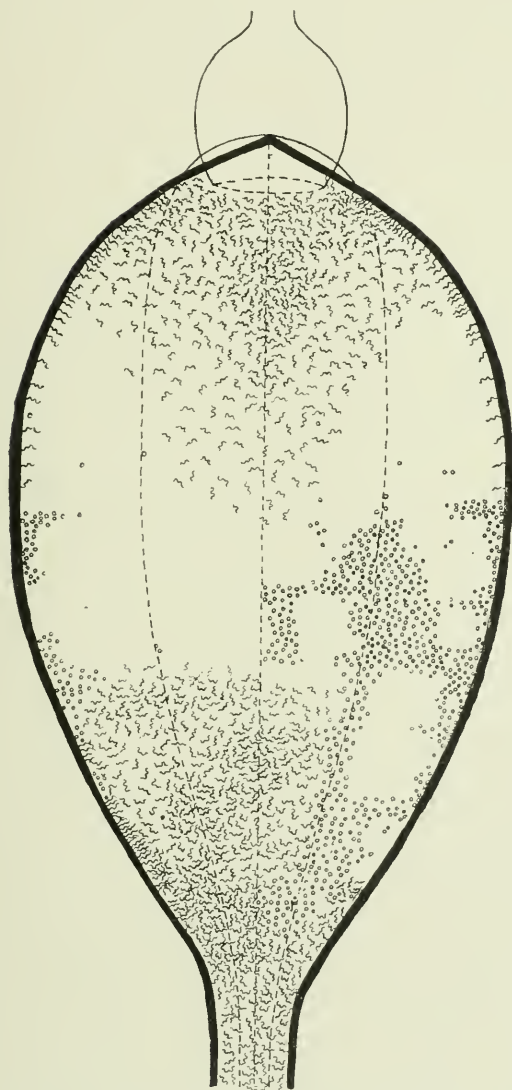
In the anterior third, approximately, of the stomach extracellular trypanosomes are abundant, both free in the debris and attached to the epithelium, but there are no intracellular stages.

In the region behind this, approximately the middle third or fourth of the stomach, there are no extracellular trypanosomes, either free or attached, but a very extensive intracellular infection is found, confined for the most part to the right side of the sections, where almost the whole of the epithelium, with the exception of the crypts, has been attacked; the reconstruction shows very well the clear spaces which represent the situations of the epithelial crypts.

In the posterior third of the stomach a remarkable condition of things is found. On the right side of the sections there are still numerous intracellular trypanosomes, but no attached extracellular parasites; on the left side of the sections there are very numerous extracellular trypanosomes, attached or in close proximity to the epithelium, but none intracellular. Scattered through the debris are many free trypanosomes which are also passing down the intestine.

Interpretation.—A stomach in which intracellular multiplication is still proceeding actively and from which trypanosomes are passing down towards the rectum.

TEXT-FIG. 12.



Reconstruction of another stomach. 24 hours. Magnified about 83.5.

(15) From a batch preserved in sublimate-acetic. A large stomach which passes through 241 sections without quite reaching pylorus.

About 10 extracellular trypanosomes, most of them attached to the epithelium, are found in the hindermost sections.

No intracellular trypanosomes are to be found.

Interpretation.—A stomach in which the intracellular multiplication is either completed or almost inhibited.

(B) The Migration to the Rectum.

As stated above, the trypanosomes occurring in the intestine are usually in transit from the stomach to the rectum, and only exceptionally attach themselves to the intestinal wall or undergo further development there. We have been able on more than one occasion to observe the actual passage of the trypanosomes along the intestine. In a flea which had fed on an infected rat about twenty-four hours previously, the stomach, with a considerable length of the intestine attached, was dissected out on a slide partly teased up and covered with a glass slip; the posterior part of the stomach, however, with the intestine attached, was left intact. Close to the pylorus the stomach contained fluid in which a great number of brown granules were suspended, coarse granules of faecal appearance evidently representing indigestible residue of the last meal. These granules could be seen to be in a state of violent commotion, more violent than could be explained merely by Brownian movement, since they were being churned and stirred in every direction; but although there could be little doubt but that the movements were due to the activity of trypanosomes, the flagellates themselves could not be seen clearly in the opaque fluid through the stomach-wall. Soon, however, a bolus of fluid passed through the pylorus into the intestine and passed down it by peristaltic action, pushed onwards like a bead, until it reached the cut end of the intestine and was extruded from it. It was then seen at once that the fluid contained, in addition to the faecal granules, a swarm of excessively active trypanosomes, long and relatively slender forms with great powers of rapid forward progression.

They began at once to spread in all directions in the fluid ; but at this moment the coverslip was picked off with needles and dropped instantly into Schaudinn's fluid ; after it had been stained and mounted it was found that, by a piece of good luck, the stomach and intestine had remained sticking to the coverslip, and that round the cut end of the intestine were several trypanosomes of the long stomach-type (Pl. 42, figs. 203, 204), evidently some of those that had been seen to pass down the intestine. In two other fleas, fed respectively twenty-four and eighteen hours previously on infected rats, we were able to confirm our observations on the passage of the trypanosomes down the intestine and to obtain preparations of them (Pl. 37, fig. 50).

It is unnecessary to give a detailed description of the migratory trypanosomes, since it is evident from the figures that they are simply of the long stomach-type already described. They are very active, and in form crithidiomorphic, with the hinder part of the body stiff and straight, sometimes slightly or even markedly clubbed and swollen. The two nuclei are more or less closely approximated, but *n* is almost always well behind *N*. It is seen from this that the trypanosomes resulting from the intracellular multiplication in the stomach may do one of two things ; they may penetrate again into epithelial cells and go through another generation of multiplication ; or they may collect in the pyloric region and be carried down the intestine (compare Pl. 45). The migration may begin as has been seen, as early as eighteen hours, but this appears to be rather exceptionally early, to judge from our observations on the rectal phase ; probably it does not usually begin till twenty-four or thirty-six hours. It continues, doubtless, as long as the stomach-phase lasts, and as already stated, we have found intracellular stages as late as five days in fleas not fed a second time ; we may suppose, therefore, that the production of the migratory forms and their passage down to the rectum, may be going on continually, in some cases, until the second feed of the flea. In other cases, however, the multiplication in the stomach probably comes to an end of itself,

before the second feed, judging by the many observed instances in which, in fleas not fed a second time, the stomach may contain many long free trypanosomes, or the rectal phase may be well established, without any intracellular multiplication occurring in the stomach.

(c) The Rectal Phase.

The starting point of the rectal phase is the long, active "crithidiomorphic" type of trypanosomes already described, which migrates down the intestine. During its passage down the intestine the changes of form and structure which may have begun already in the stomach continue, so that by the time it reaches the rectum its posterior end is generally distinctly club-shaped. Arrived in the rectum, it very soon undergoes changes in form, habits and structure, and multiplies by binary fission, giving rise ultimately to the typical forms of the established rectal phase, forms which, apart from other characters, are of much smaller size and bulk than those of the stomach-phase. We will discuss first (*a*) the transition from the initial to the established forms of the rectal phase, and then (*b*) the various types of modifications of the latter, culminating in the little trypanosome-form, which is the final stage of the development of the flea.

(*a*) The Transition to the Crithidial Form.

If the various processes of change in the initial stages of the rectal phase be analyzed, after study of both living and preserved material, and by comparing the starting point of this part of the development with its final result, we may note the following tendencies in the organism.

In the first place it loses its intense activity and becomes more sluggish in movement, with a great tendency to attach itself by the tip of the flagellum; under natural conditions the flagellate attaches itself to the wall of the hind-gut, but when under microscopic examination it can be seen to adhere

firmly to pieces of débris of any kind, or to the surface of the glass slide or coverslip. When not attached in this way it progresses slowly with the flagellum directed forwards.

Secondly, the body shortens and changes in form by the cytoplasm becoming concentrated towards the posterior end of the body.

Thirdly, the flagellum becomes progressively shortened.

Fourthly, the two nuclei, if still in their original positions, become transposed into the typical crithidial arrangement, with *n* close beside or in front of *N*.

Fifthly, the nuclei, the flagellum, and finally the whole body are multiplied by division or reduplication.

The order in which these different processes of change have just been stated is in no way to be taken as indicating their chronological order of succession in the development, since they take place more or less independently and, as it were, at different rates of acceleration in different individuals; the result is consequently the production of a great number of forms which at first are rather bewildering and difficult to arrange in a series. The difficulty of tracing in detail the transition from the initial stage of the rectal phase to the established crithidial condition is increased by the fact that the early transitional stages are extraordinarily rare and difficult to find in the permanent preparations; a fact which indicates that the transition takes place very rapidly and is completed very quickly. One explanation of this rapid change may perhaps be found in the great diminution in size which is brought about in this part of the development. Leaving out of consideration for the moment any structural or other changes, it is at least quite clear that the large individuals which come down from the stomach initiate a series of generations of multiplication by fission culminating in forms perhaps not more than a tenth the size of the initial forms from which they are derived. Consequently it is probable that the successive divisions of the body follow one another at first with extreme rapidity and without intervening pauses to allow the daughter-individuals

to grow to the size of the parent, as would happen in the normal multiplication such as takes place in the established crithidial stage. Another explanation for the rarity of the transition-stages may be the possibility that of the long trypanosome-forms which come down from the stomach to the rectum, only a small number may go through their metamorphosis into the typical rectal phase and the greater number may degenerate or be carried out of the flea. Whether this be true or not, it is not necessary to suppose that all those which pass down from the stomach do so in a single swarm; it is more probable that they dribble down from the stomach, so to speak, in larger or smaller bands or singly, a supposition which would also account for the small number found at any one time in the rectum undergoing their metamorphosis.

A further difficulty which may arise in distinguishing the forms of the transition from trypanosome to crithidia, and in assigning them to their proper position in the series, is the fact that the body may become artificially broadened in preparations which have been allowed inadvertently to dry up the least bit before fixation. Deformed specimens of this kind can be recognized by their flattened appearance and consequent even staining of the body, which in a properly preserved specimen should be thicker and more opaque in the axial region than towards the edges of the body; the trophonucleus in flattened specimens is often transversely elongated; and, further, the process of drying seldom affects a single specimen, unless it is very isolated or near the edge of the film, but, if it has taken place at all, the effects of desiccation are apparent over at least a considerable area of the slide or coverslip. It is, therefore, not difficult with a little practice to detect the specimens which have become broadened artificially; and in any case the process of drying does not affect the length of the body or flagellum to any appreciable extent.

It is a result, doubtless, of the rapidity with which the transition is effected that we have not been able to come to a perfect agreement of opinion between ourselves as to certain points of the development during this transitional period,

namely, the exact stage in the process of change of form at which the first division of the initial rectal form (that is to say, the long club-shaped forms that come down from the stomach) takes place, and consequently the type of binary division, whether equal or unequal, which initiates the whole series of generations in the rectal phase. Before we discuss this doubtful point, we may first classify the various types seen in the initial transitional stages of the rectal phase, for which it is simplest to take the types of body-form as the basis of classification. Bearing in mind the considerations of technique that have been discussed already, and being careful, therefore, to eliminate all cases where there is reason to suspect artificial deformation of the specimens, we can recognise the following series of forms, each of which is a stage in the progressive shortening and broadening of the body by concentration of the cytoplasmic substance in the posterior third of the original slender trypanosome:

(a) Slender forms, differing but little from the long stomach type, and evidently but recently arrived in the rectum. The flagellum is still long, and *N* is usually in front of *n* (Pl. 42, fig. 208); sometimes, however, the reverse is the case (Pl. 42, fig. 209).

(b) Forms in which the hinder region of the body, containing the two nuclei, become swollen and club-shaped (Pl. 42, fig. 206), and the anterior part correspondingly attenuated, until the body as a whole becomes more or less tadpole-shaped (Pl. 41, fig. 150; Pl. 42, figs. 205, 207). The flagellum at this stage is usually long, but sometimes distinctly shortened (Pl. 41, fig. 158; Pl. 42, fig. 210).¹ The nuclei are usually still in their original relative positions, but are sometimes crithidial in arrangement (Pl. 42, fig. 211).

(c) Forms in which the concentration of the body-substance

¹ How the shortening of the flagellum takes place is not clear, but it is worthy of note that in smears there are often found broken pieces of flagella near the specimens, as if the flagellum had become brittle and had broken off (Pl. 41, figs. 158, 160).

in the posterior half or third of the body has proceeded so far that the body is nearly half as broad as long, and the anterior prolongation which forms the undulating membrane is much reduced in length and very slender. Examples of transitions from the last stage to this are seen in Pl. 41, figs. 151, 152 ;

TEXT-FIG. 13.

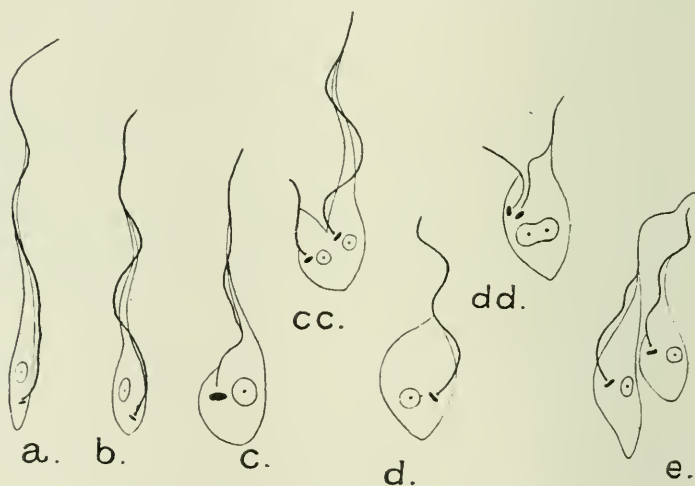


Diagram to show the possible modes of transition from the stomach-phase to the rectal (crithidial) phase: *a*, slender form newly arrived from the stomach (compare Pl. 42, fig. 208, etc.); *b*, early club-shaped form (compare Pl. 42, fig. 205, etc.); *c*, later (more swollen) club-shaped form (compare Pl. 41, fig. 149); *cc*, unequal division of *c* (hypothetical); *d*, large pear-shaped form (compare Pl. 41, figs. 154, 155, etc.); *dd*, division of *d* (compare Pl. 41, fig. 179); *e*, forms resulting from the initial division of the rectal phase (compare Pl. 41, figs. 153, 157, 164, etc.). (× about 2000.)

the complete realisation of this stage is seen in Pl. 41, figs. 149, 154, 162 ; Pl. 42, fig. 212. From a comparison of the figures it is seen that the position of the nuclei varies considerably ; *n* may still be at or near the hinder end (Pl. 41, fig. 162), or may be close beside *N* (Pl. 41, figs. 149, 154 ; Pl. 42, fig. 212). The hinder end may be pointed, or quite round. The

flagellum may still be a fair length, or quite short (Pl. 41, fig. 162).

(d) Forms in which the body is contracted into a pear-shaped mass, nearly as broad as long. The stalk of the pear is formed by the flagellum together with a slight projection of the body representing the anterior prolongation, now usually reduced to its smallest limits (Pl. 41, figs. 155, 156, 161; Pl. 42, fig. 215). The nuclei are usually transposed, *n* being beside or in front of *N*. The length of the flagellum varies within wide limits represented by Pl. 41, figs. 155 and 156.

(e) Forms which are distinguished from the preceding stage chiefly by their smaller size. Here again, however, caution is necessary in referring a given specimen to its place in the series, since the apparent size in permanent preparations may be affected considerably by technique. In the preparations fixed on the slide with osmic vapour and stained by Giemsa's method the trypanosomes always appear considerably larger than those on the coverslips fixed wet with sublimate solutions, stained with hæmatoxylin and mounted without drying in canada balsam (vide Minchin, 1909). Consequently, different standards of sizes are required for interpreting preparations made by these two different methods of procedure, and preparations made by the one method must not be compared directly, without making due allowance for the differences in result, with those made out by the other method. Nevertheless, after giving due weight to these considerations, it is not difficult to distinguish forms which are about half the bulk of the stages already described (Pl. 41, figs. 153, 159, 160, 164; Pl. 42, fig. 218). Such forms are, without doubt, individuals derived from at least one, possibly more than one, division of the initial form, a conclusion supported by the occurrence of such forms in pairs, possibly as the result of division recently completed (Pl. 41, fig. 157; Pl. 42, fig. 216*a*).

The question which must remain open at present is, in which of the four stages of changes of form (*a*), (*b*), (*c*), or

(*d*) (see Text-fig. 13), does the initial division of the rectal phase take place?

If, as is possible, and as one of us (J. D. T.) thinks probable, the first division takes place in the club-shaped form as soon as the concentration of the protoplasm is completed and when (as in Text-fig. 13, *c*) the two nuclei lie close together, it may well be the case that the products of division would then be markedly unequal in appearance owing to the retention of the old flagellum by one of the two daughter-individuals; that is to say, the division would be of the type found in similar club-shaped forms in the trypanosome of the gold-fish both in cultures and in the leech, and in bird-trypanosomes both in cultures and in the mosquito, and which has also been seen in an early culture of *T. lewisi* itself. On this view the swollen, pear-shaped forms of stage *d* would have to be interpreted as forms subsequent to, and the products of, the initial division. In spite of much searching, however, through our preparations, we have been unable to find actual examples of club-shaped forms showing division markedly unequal in appearance.

If, on the other hand, as one of us (E. A. M.) believes, it is the most usual state of things for the organism to continue the process of contraction and broadening of the body until it has reached the condition of stage *d*, it is probable that the flagellate would then divide by the type of binary fission characteristic of the subsequent generations of the crithidial phase, that is to say, producing two daughter-individuals that are equal, or not markedly unequal in bulk, and differing only in that one of them has the flagellum temporarily shorter than that of the other. Pl. 41, figs. 179, 180, and Pl. 42, fig. 216, may then be interpreted as examples of the initial division, and Pl. 41, fig. 157 and Pl. 42, fig. 216 *a*, as pairs resulting from the initial division recently completed.

The problem of the initial division of the trypanosomes in the rectum is one which involves more than the question as to the type of form, club-shaped or pear-shaped, in which the division takes place, or the question whether the products of division differ in visible characters of

bulk, structure, or appearance; it raises a much deeper and more fundamental problem, namely, whether the division which initiates the rectal phase is an equating division which gives rise to two equipotential daughter-individuals or a differentiating division which produces inequipotential forms. If the division-products are equipotential, then visible differences between them of any kind would be merely temporary and of no significance for the future behaviour and destiny of the sister-individuals, which would be true twins; all such differences, however pronounced, would be immaterial for the development as a whole, and the same would apply to the parent individual, whether club-shaped or pear-shaped. If, on the other hand, the daughter-individuals are inequipotential, in the sense that the smaller of the two division-products becomes the starting point of an indefinite number of generations of small crithidial individuals, while the larger is merely a "parent" which, though it may divide in the same manner to produce small crithidial forms several times in succession, does not ultimately develop further, but drops out, as it were, of the direct line of the development when its powers of reproduction are exhausted; if they are inequipotential in this sense, it follows that the observed difference between the two daughter-individuals would have an important significance and that they would not then be true twins, and further, that the club-shaped form, assuming that this is the form in which division takes place, would then be a developmental form of special and peculiar significance in the life-cycle and not merely a stage in the change of form leading to the pear-shaped stage. We must be content, unfortunately, with enunciating these possibilities, without being in a position to decide between them; owing to the fact, to which reference has been made above, that the extreme rarity of transitional forms in our preparations has supplied us with insufficient material for a decisive judgment.

The type of the initial division and the exact point at which it occurs in the series of progressive form-changes of the rectal phase must be left at present an open question, unfortunately; but this much may be stated positively about the process of division in the rectal phase in all cases, whether in the initial or subsequent stages. No multiple division occurs henceforth in the developmental cycle, but the parasites settle down to a course of simple binary fission continued indefinitely, and always taking place in the lumen of the gut, never within cells. The process of fission is initiated by division of the blepharoplast or basal granule of the flagellum, but the flagellum itself does not divide; the original or parent

flagellum remains attached to one of the two daughter-blepharoplasts and a new flagellum grows out from the other blepharoplast. The division of n follows hard upon that of the blepharoplast, then N starts its division, and finally the whole body divides; of the two daughter-flagellates produced, one has the original flagellum, the other has to grow a new one, which it may not do, in some cases, until after complete separation from its twin sister. In any case, one of the two products of division has a much shorter flagellum than the other, irrespective of any difference in bulk between the two.

As already stated, while these changes of form and processes of multiplication are going on the flagellate is also undergoing a change which, though a very small thing in itself, produces nevertheless the characteristic morphological distinction between the trypaniform and crithidial types; namely, the approximation and final transposition of the two nuclei, so that n comes to lie beside, or even well in front of, N . The approximation of the two nuclei begins with the first alteration and changes of form, but the exact point in the development at which the change of position of the two nuclei is completed is subject to great variation. Very exceptionally, as has been described above, the transposition of the nuclei may be complete in the stomach itself, but more usually, it may be said normally, the change does not take place until the flagellate reaches the rectum. Any of the forms distinguished above as a , b , c , d or e , may be found with the nuclei, but slightly or completely approximated, and finally in the clumps of developmental forms such as that represented in Pl. 41, figs. 182-184, evidently consisting of forms which have completed several, at least two or three generations of fission since arriving in the rectum, all stages in the process of transposition of n or N are to be observed. Since the small forms of the established rectal phase show invariably the typical crithidial arrangement of the nuclei, all that can be said is that the change in position of the nuclei is effected earlier or later, but without fail, in the transition from the stomach-phase to the rectal phase.

The successive generations of the transitional rectal phase cannot be distinguished with precision. The size to which the individuals are diminished after a given number of generations is determined by two variable factors during those generations, namely, the rate of individual growth and the frequency with which multiplication takes place. All that can be said is that the rectal forms diminish in size by repeated division until they reach a minimum size which is attained when growth and multiplication balance each other more or less evenly; and that in the rectal infections of recent origin the average size of the individual flagellates is slightly larger, as a rule, than in the old-established infections.

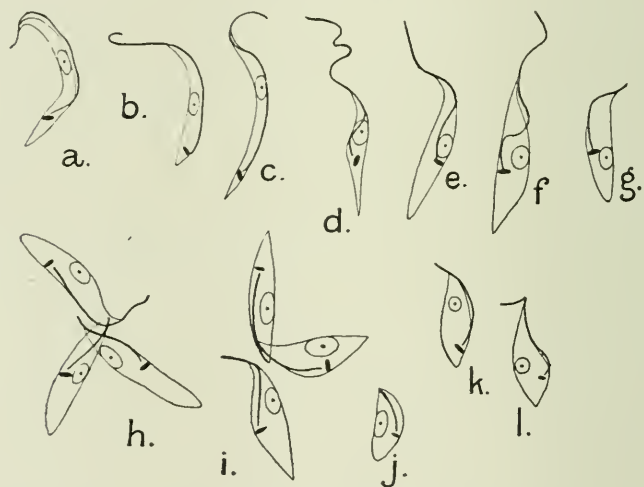
(b) The Established Rectal Phase.

In its fully-established and definitive condition the rectal phase consists chiefly of small crithidial individuals which multiply by binary fission, attached to the wall of the gut by their flagella or by the anterior (flagellar) extremity of the body. The crithidial stage appears normally, if not invariably, to take origin in the rectum in the manner described in the preceding section, and this part of the digestive tract is its most usual and characteristic habitat; consequently we have made use of the term "rectal phase" for this part of the development, in spite of the fact, presently to be discussed in more detail, that the crithidial and other forms of this phase may be found not uncommonly in other parts of the gut, more especially in the intestine close behind the pylorus, but also in the stomach itself.

In one flea we have found the rectal phase well-established so early as eighteen hours after the infective feed (Text-fig. 14), and in another flea we found a few typical examples of this phase at twenty-four hours, but both these examples are abnormally early cases of the fully-developed crithidial phase, which, in our experience, is seldom completely established before thirty-six or forty-eight hours after the infective feed.

The extent to which the crithidial infection is developed in different fleas varies greatly, from a swarming infection covering the wall of the rectum like a pile-carpet, especially in the region behind the rectal papillæ, to a condition in which a few scanty flagellates are to be found only by careful searching; in either case, however, the crithidial stage represents

TEXT-FIG. 14.



Various forms from the rectum of a flea eighteen hours after the infective feed. *a*, *b*, *c* and *d* are probably degenerative forms, the rest developmental; *e* and *f*, early rectal forms, *g-l*, haptomonads ($\times 2000$).

the permanent stock of the parasites in the flea, multiplying continually and indefinitely and maintaining itself, in all probability, as long as the flea lives. We have succeeded in keeping a single flea alive for nearly three months, and during that time seven rats were infected by it (see p. 640 below). This permanent infectivity can only be explained by the establishment of the crithidial stock in the rectum and its continued multiplication. Each crithidial individual may do one of two things: it may multiply by binary fission to produce two crithidial forms (multiplicative phase); or it may be

transformed gradually into small stumpy trypaniform individuals (final propagative phase). Hence the flagellates of the rectal stock may be classified, broadly-speaking, into crithidial, transitional and trypaniform individuals.

The forms that occur in a well-established rectal infection are very varied, and it is a somewhat difficult task to classify them and to assign to each and every form its due place in the developmental series. To obtain a general notion of the various types met with, it is best to examine fleas some eight or nine days after they have fed once on an infected rat and subsequently on clean rats, so as to obtain the rectal phase in its typical condition, free from admixture of earlier developmental or degenerative forms. Such fleas may have no flagellates at all in their rectum or may present every gradation between an extremely scanty and a swarming infection.

If a rectum containing numerous flagellates be examined fresh after having been dissected out in salt-citrate solution, opened up by tearing it with fine needles, and then covered with a coverslip, the majority of the flagellates are seen attached to the wall of the rectum, chiefly in the region behind the rectal papillæ. Many of them will have become detached as the result of the dissection, and will be seen floating about singly or adhering together in larger or smaller clumps. If the preparation be sealed up carefully and watched for some time many of those that lie about singly will attach themselves to the glass of the slide or coverslip, and it can be seen very clearly in all cases, whether in the forms still attached to the rectal wall or in those set free in clumps or singly, that the attachment is by the tip of the flagellum or by the flagellar extremity of the body in those that have no free flagellum. The flagellates may be attached to the rectal wall in such numbers and so closely crowded together that they resemble a furry lining or pile on it. Seen in profile they appear in serried ranks, each in contact with its neighbours; seen in surface view they present the appearance of a honeycomb, each flagellate in optical transverse section, showing outlines nearly polygonal as the result of mutual pressure. In the living condition the attached

crithidias are motionless for the most part, but occasionally a given individual performs a kind of jerky nutating movement; the body, remaining attached, sways rapidly from one side to the other, with a slight curvature of the axis. After bending, first to one side and then to the other, in this way the animal remains quiescent for a time, but when there are a large number of the flagellates, there is scarcely a moment in which one or another, or several at once here and there, are not performing these movements.

In addition to the attached forms, with the flagellum for the most part very short or wanting altogether, there are usually a certain number of free forms. Some of these are crithidial in form, with the undulating membrane feebly developed and scarcely, if at all, recognisable, and with a distinct free flagellum, often quite long. The function of these flagellated forms appears to be that of migrating in order to colonize other parts of the wall of the gut. When a clump of attached forms has been multiplying actively at one spot it doubtless tends to become overcrowded, at least in its central part. Then probably one of two things may happen; some of the crithidias may become transformed into the final trypanosome-form and detached from the clump; or a certain number will develop flagella, remaining crithidial in type, migrate to another part, and attach themselves again.¹

Besides the crithidial forms with long flagellum there can be seen free forms, also with a flagellum of variable length, but with a distinct undulating membrane running along the whole or greater part of the length of the body, which appears more flexible and performs sinuous undulatory movements more or less distinctly. These forms are the little trypanosomes, or transitional stages in their development, the forms which constitute the final infective stage of

¹ One of us has seen in the living rectum a crithidia with a long flagellum become detached from the rectal wall, swim actively across the rectal cavity, with its flagellum directed forwards, and attach itself again to the wall on the side opposite to its former attachment.

the cycle in the flea (so-called "metacyclical trypanosomes" of Brumpt).

We can thus distinguish in the rectum of the flea three principal types of individuals, each of which varies considerably in size and details of form, and between which are to be found every possible transition, namely :

(1) The attached or haptomonad¹ form, which is the multiplicative stage of the rectal development.

(2) The free or nectomonad¹ form.

(3) The final trypanosome-form.

The many variations of these three types and the transitions between them cause the rectal phase of the development to present a variety of form which is at first very bewildering, but which becomes easily intelligible if the classification into the three types given above be used as the key. It may be noted here that it is very common, in the development of other trypanosomes, for the crithidial phase to exhibit great variation in size, form, and structure. Compare the works of Chagas on the development of *T. cruzi* in the bug, and of Miss Robertson (1911, etc.) on the development of the trypanosomes of fishes in leeches.

We will now proceed to describe the variations of these types and the transitions between them in more detail.

(1) The haptomonad or attached type is the phase in which multiplication by binary fission takes place ; consequently its variations of form are related mainly to the function of multiplication. Leaving out the forms which are actually in process of division, we find that chief variations in form are seen : first, with regard to the shape of the body, whether relatively slender, with the hinder end sharply or bluntly

¹ Woodcock (1914) has proposed the useful term "haptomonad" for the attached phase of a *Crithidia* or *Leptomonas*, the so-called "gregariniiform" individuals of Léger, but of which the resemblance to a gregarine is not very striking, not at least in the developmental phases of *Trypanosoma lewisi*. To denote the locomotor crithidias with long flagella we propose the term "nectomonad," i. e. swimming monad, as a correlative to haptomonad, fixed monad.

pointed, or stouter, with rounded hinder end, or finally ovoid or even globular in form; secondly, with regard to the extent to which the flagellum is developed.

The typical haptomonad, when not preparing for division, has the body spindle-shaped or pear-shaped with the thickest part anteriorly in front of the nuclei, and the hinder end more or less sharply pointed (Pl. 41, figs. 174, 176, 193; Pl. 42, fig. 252). The two nuclei are usually close together, situated either about the middle of the body or nearer to the hinder end; *n* is either just in front of *N* or close beside it. The cytoplasm has a great tendency to stain very dark by any method, especially towards the hinder end. After Giemsa the body has a purplish-blue tinge; after Twort's combination of neutral red and Lichtgrün it is seen to be full of very fine granules, stained red and scattered irregularly (Pl. 38, figs. 260a-263a); from these reactions it is evident that the opacity of the body is due to the deposition in the cytoplasm of very fine "chromatoid" grains, probably of the nature of volutin. The cytoplasm is usually free from coarse granulations, which are, however, present occasionally.

In preparation for division the hinder end of the body begins to swell and to become rounded at the hinder end, while the nuclei are shifted more posteriorly. In consequence the body becomes pear-shaped, but in the opposite manner to that previously described, since now the thickest part of the pear is the posterior end, while the anterior extremity of the body is narrowed, with the flagellum representing, as it were, the stalk of the pear (Pl. 41, figs. 168, 188; Pl. 42, figs. 231, 247-249). In other cases the body becomes evenly ovoid or even globular in form (Pl. 42, figs. 243-247).

The process of division calls for no special remark (see Pl. 42, figs. 213, 214, 226-230, 249, 253, 266). The blepharoplast or basal granule of the flagellum divides first, one of the daughter-blepharoplasts retaining the old flagellum attached to it, while the root of the new flagellum begins to grow out from the other daughter-blepharoplast (Pl. 42, fig. 252, lowest specimen). Following the blepharoplast, *n* divides

next and after that *N*. Next the body is constricted into two, beginning from the anterior or flagellar end (Pl. 42, fig. 253). We have never seen any but binary fission of the crithidial forms; multiple fission does not occur in any form at this stage. We have also sought without success for multiplication by endogenous budding (the so-called "infective granules" of Balfour and others). In *Leptomonas pattoni* of the flea we have found very clear instances of apparent endogenous budding (Pl. 42, figs. 281, 282-284), and Pl. 42, figs. 268, 269 are rather suggestive of a similar process occurring in the haptomonads of *T. lewisi*, but further proof of it is lacking.

The flagellum in haptomonad forms is extremely short, and as a rule is reduced practically to its intra-cellular root or rhizoplast, the projection beyond the limits of the cell-body being very slight or quite imperceptible. At the point where the rhizoplast comes to the surface at the anterior apex of the body there is usually a fairly large and distinct, but often ill-defined patch of substance which stains like the flagellum, that is to say, red, after Giemsa's stain, black or greyish-black after iron-haematoxylin, and green with Twort's stain, and which appears to represent a secretion produced by the flagellate, a sort of cement by which the animal adheres to the wall of the rectum (Pl. 41, figs. 174, 178; Pl. 42, figs. 223, 226, 243-245, 260, 262, etc.).

In many cases the rhizoplast fails to reach the surface of the body, or may even, very exceptionally, be absent altogether (Pl. 42, figs. 241-243, 267). The body is then always ovoid or globular in form. In such cases we have true leishmanial forms, which appear to owe their origin to very rapid multiplication of the ordinary haptomonad type; multiplication is so rapid that one of the two daughter-individuals resulting from binary fission has no time to form completely its new flagellum or even the rhizoplast, before being split off from its twin sister and beginning to divide again. This view receives support from the extremely small size which is commonly a feature of these leishmanial forms. In no case have we seen

anything that could be interpreted as encystation or encapsulation of the leishmanial forms; they appear to represent a purely trophic and multiplicative phase.

The haptomonad forms occur usually, as has been said, attached to the cuticle lining the rectum; in surface views of the rectal wall, living or preserved, they may be seen attached singly, in clumps, or in a continuous carpet-like layer, and exactly the same is found in microtome-sections of preserved recta (Pl. 44, fig. 317; Pl. 42, fig. 277). In sections of a well-infected rectum the haptomonad forms are seen in a long, continuous line, like soldiers on parade. But, both in living and preserved recta, in film preparations or in sections, free clumps of haptomonads are also found, in which the individuals all have their flagella directed toward a certain point (Pl. 42, fig. 274). In spite of careful scrutiny it is not possible to detect any body or particle of débris at the centre to which the monads are attached; they appear to adhere simply to one another by their flagellar extremities. The question at once arises whether these free clumps are a natural or an artificial condition. If they were only seen in teased-up recta one could have hesitation in ascribing their detachment to the manipulation, but they are found also in sections of recta. It is, of course, impossible to dissect out and preserve a rectum without subjecting it to great stresses and strains which might detach the monads, but it is remarkable how tenaciously they adhere to the wall. In one of our series of sections of a rectum it can be seen that it has been badly torn in getting it out; part of the torn wall has curled right back and turned inside out. The tear goes right through an attached carpet of crithidias which have nevertheless remained adherent to the wall, even on the part that has curled back, giving at a first glance the erroneous impression that the monads are attached to the exterior of the rectum. They must, therefore, be attached very firmly to the wall, which is intelligible when it is recognised that the crithidial forms are not the ripe, propagative stages of the cycle and that if they were carried to the exterior with the fæces they would be lost. For this

reason alone it seems highly probable that the free clumps of crithidial forms represent either clumps artificially detached by manipulation, or an abnormal condition of the flagellates detrimental to their future welfare.¹

As transitions between the nectomonad and haptomonad phase we would expect to find both stages of the development of the nectomonad into the haptomonad, and stages of the development in the reverse direction. It is of course almost impossible to say, by simple inspection of a transitional form in a permanent preparation, in which direction it is developing. We are inclined, however, to interpret as transitions from the nectomonad to the haptomonad the more slender forms, with short flagella and hinder ends pointed or but slightly blunted, such as Pl. 41, figs. 175, 176; Pl. 42, figs. 220, 252; and as transitions in the opposite directions the rounded or broad pear-shaped forms with flagella of various lengths, such as Pl. 41, fig. 166, Pl. 42, figs. 231-234, 251, 265. In some forms of the latter type the distal ends of the flagella are very thin, much thinner and more delicate than the proximal portions (Pl. 42, figs. 246, 250, 268, 269), and we interpret this appearance as indicating that the flagellum is in process of growth rather than of regression in length, for the reason that a similar condition is seen in the flagella of forms transitional to the final trypanosome-type (Pl. 42, figs. 255-257), in which the flagellum is not likely to be in process of shortening.

In the series which we interpret as transitional from the haptomonad to the nectomonad type we find globular forms with flagella of considerable length (Pl. 41, fig. 166; Pl. 42, fig. 234), and, since we have not observed such forms swimming freely in the rectum, we conclude that the haptomonad, while attached, first develops its flagellum to a considerable length, and then acquires the elongated form of body, before becoming detached from the wall and set free.

¹ Comparable, for example, to the sponge-larvæ, which attach themselves to the surface-film of the water instead of becoming fixed to a firm object, and which in consequence perish inevitably.—E. A. M.

(2) The nectomonad or free type of crithidial flagellate has a more slender body, in its fully-developed form about five times or more as long as its greatest breadth, but with great variations in its relative proportions (Pl. 41, figs. 190, 194-197; Pl. 42, figs. 217, 235, 236, 254). The body is usually spindle-shaped, pointed at both ends, its thickest part at the level of or slightly behind, the middle point of its length, and *n* is usually well in front of *N*. When it swims the flagellum, directed forwards, is thrown into even sinuous undulations which begin at the tip and run backwards, in contrast to the type of movement so often seen in free-living flagellates, in which the proximal two-thirds, or so, of the flagellum is held stiff and straight, while the distal third performs lashing movements which drag the body forward. We have not found the fully-developed nectomonad type undergoing multiplication by fission, unless Pl. 42, figs. 253 and 266 are to be so interpreted.

(3) The final trypanosome-form appears to rise in most cases from the haptomonad type, with which it is usually found closely associated in preparations; compare Pl. 41, fig. 202, of a section through the intestine close behind the pylorus; the trypanosomes are seen with their posterior ends projecting above the level of the serried ranks of the haptomonad crithidias, as if they were pushed upwards by the development and growth of their flagella. It is possible, however, that the final forms may sometimes arise from the nectomonad type, and that such an origin explains the occurrence of the slender forms of the trypanosomes, the stout forms being derived from the haptomonads. Forms such as Pl. 42, fig. 237, are perhaps to be interpreted as transitional from the nectomonad type to the final trypanosome-form.

The essential feature in the origin of the final form from the crithidial form, of whatever type, is the transposition of the two nuclei, *n*, and *N*. Both nuclei move backwards usually, but *N* only for a short distance, while *n* passes *N* and goes to the posterior extremity of the body (Pl. 42, figs. 238, 239,

255-259, 270). In some cases, especially in the slender forms, n stops short of the extreme posterior end of the body (fig. 239), but in the stumpy forms n becomes quite terminal in position, as a rule (Pl. 41, figs. 199, 200; Pl. 42, figs. 259, 271). Further characteristic of the final stage is the relatively large size of both n and N , and the faint stain that N usually takes in the permanent preparations. In many cases N appears distinctly elongated in the longitudinal direction (figs. 199, 259). With the displacement backwards of n and of the attachment and origin of the flagellum, the undulating membrane becomes correspondingly extended and lengthened.

The occurrence of stout and slender forms of the final trypanosomes has been mentioned already, and was pointed out by Swellengrebel and Strickland (1910); but it is a fact somewhat difficult to explain. It may be, as already suggested, that it is simply due to difference of origin, the slender forms arising from the nectomonads, the stout forms from the haptomonads. On the other hand, it may be that the trypanosomes, when ingested by the rat, become exceedingly active in order to find their way from the digestive tract into the blood, and that the slender forms in the flea represent merely the precocious assumption of a type of structure which belongs strictly to a later period of the life-cycle. These are the only suggestions we can offer at present in explanation of the two forms.

We have never in any case seen the final trypanosome-form dividing, but it is stated to do so by Swellengrebel and Strickland (1910), who, after having examined one batch of thirty-seven infected fleas, have been able to figure no less than three examples of a process of division that we have never been able to find in all the many hundreds of infected fleas we have dissected and examined. For our part we agree with Brumpt (1913), that these "metacyclical trypanosomes," as he proposes to call them, are "phases d'attente" which do not multiply further in the flea.

With the development of the final trypanosome-form the

cycle of *T. lewisi* in the flea is ended. It only remains to say a few words with regard to the occurrence of the rectal phase in regions of the gut situated further forwards than the rectum. It is by no means an infrequent occurrence to find clumps and carpets of various forms characteristic of the rectal phase attached in the intestine and even in the stomach.¹ In the intestine they occur most frequently at the upper end, close behind the pylorus. When they occur in the stomach they are probably always attached towards its hinder end, near the pylorus. Hence the two chief situations of the crithidial forms, when occurring outside the rectum, may be designated briefly "pre-pyloric" and "post-pyloric."

Two possibilities present themselves at once to the mind with reference to these extra-rectal crithidial infections; first, that the infection of the stomach or intestine is a direct one, brought about by forms which have attached themselves there immediately after completing their stomach-phase, without having ever travelled further back in the digestive tract; secondly, that the infection has been brought about in an indirect manner by forms which have migrated forwards from the rectum.

So far as post-pyloric intestinal infections are concerned, we have some evidence that the infection may be sometimes a direct one; in one of our series of sections of the stomach of a flea that had fed thirty-six hours previously to being preserved, there are two large clumps of crithidial forms attached close behind the pylorus. Probably in such cases those attached in the intestine represent but a small numerical proportion of those that migrated backwards from the stomach, the majority having passed on down to the rectum, while a few have stuck, as it were, higher up.

With regard, however, to the pre-pyloric crithidial infections, we have no evidence of direct infection taking place, but all our data indicate that such infections of the stomach are

¹ Since a certain length of intestine was usually cut off with the stomach it is possible that many of the crithidial forms found by us in our stomach-films were really post-pyloric in situation.

brought about indirectly, and the same is probably true, in most cases, of the post-pyloric infections of the intestine. In the first place we have no record of the occurrence of crithidial infections in the stomach (pre-pyloric) earlier than seven days after the first infective feed of the fleas; but at later periods than this we have so many records of such infections in the stomach that, had they been in all cases brought about directly, we should have expected to have found crithidial forms in the stomach during the period when such forms are being established, that is to say, from about thirty-six hours and five days or so, which we have never done. Secondly, the evidence furnished by experiment 39 (see below, p. 634), indicates very strongly that the final infective forms of the life-cycle were first produced in the rectum on the fifth day and were there also on the seventh day, but had migrated forwards to the stomach on the tenth day.

It seems, therefore, most probable that in the majority of cases at least, the pre-pyloric and even the post-pyloric infections are the secondary results of a migration forward from the rectum of crithidial forms previously established there; and since neither the haptomonads nor the final trypanosome-forms appear capable of undertaking such migrations, it must be the nectomonads, which are obviously active locomotor forms, that are responsible for such migration. We have performed some experiments from which it is clear that the migration forwards is dependent on conditions of nutrition in the flea and that starvation favours a forward migration of the nectomonads towards the stomach.

In many cases the forwards migration of the flagellates leads to the rectum being quite deserted by them. This is well shown by the following instance, by no means an isolated one of its kind in our experience, but very typical. A flea was taken from the infected breeding-cage and put by itself on a clean rat for three days, from the 19th to the 22nd of September; it was then recovered and dissected. The stomach-preparations were found to contain a considerable infection of the typical rectal phase (Text-fig. 17, p. 627),

but no flagellates of any kind were found in the rectum. The rat became infected, and first showed trypanosomes in its blood on September 28th. The age of the infection of the flea was not known, but the crithidial stock seems in this case to have died out in the rectum and to have established itself exclusively in the pyloric region.

We have also, though rarely, seen the attached crithidial form in the proximal portions of the Malpighian tubules.

On the other hand we have never seen in our rat-fleas (*Ceratophyllus fasciatus*) infections such as are described by Nöller (1912), and Wenyon (1913), in the dog-flea, where both the rectum and the intestine are described as being carpeted along their whole extent with the crithidial phase; though we have seen such infections in fleas harbouring the *Leptomonas*. It is a fact which seems at first strange, but is probably very significant, that, as we have pointed out elsewhere (p. 610), the rat-flea is not so efficient a host for the rat-trypanosome as other species of fleas which do not usually or of choice feed upon rats; from which circumstance it would appear as if the rat-flea has acquired a certain degree of natural immunity to the trypanosome of the rat which other fleas do not possess.

For a general summary of the development of *Trypanosoma lewisi* in the flea, see Plate 45, and the description of it (p. 691).

(3) THE DEGENERATIVE SERIES.

Trypanosomes undergoing degenerative changes may be found in either the stomach or rectum during the first few days after the flea has fed for the first time on an infected rat. They are most abundant in batches of fleas examined during the first twenty-four hours after feeding. After this period trypanosomes may have disappeared altogether from the gut of the flea, and after thirty-six hours degenerative forms are of infrequent occurrence. In some cases, however, degenerative forms may be found in the rectum much later

than the first day, namely, up to three, four, or even five days after the infective feed. The degenerative forms of late occurrence are probably to be interpreted as individuals which have become degenerative after having developed in a normal manner for a longer or shorter period. The trypanosomes which begin to degenerate immediately after being ingested by the flea probably do not last long beyond twenty-four or thirty-six hours, usually not so long. Fleas that have fed on an infected rat whose blood is swarming with trypanosomes often show no trace of the parasites in any part of the gut by twenty-four hours. The majority of the degenerative trypanosomes that are found in the fleas are those that begin to degenerate immediately after being taken up from the rat.

There is no essential difference between the degenerative forms found in the stomach and the rectum. We may, therefore, give a general description of the forms of the degenerative series without taking special note of their provenance.

In direct contrast to the changes undergone by the developmental forms in the stomach, the principal sign of degeneration is a progressive diminution in size, more especially in the length of the body. The trypanosome gradually dwindles and wastes away, beginning at the flagellar end, during which process the flagellum becomes converted progressively into a fluffy mass, which frequently shows a tendency to stain blue or bluish with the Giemsa stain, instead of the normal red (Pl. 43, figs. 294-296, 308). Meanwhile *N* is pushed backwards towards *n*. The displacement of *N* does not appear to be due to any active migration on its part, but to be the purely passive consequence of the dwindling of the anterior part of the body, whereby it is forced backwards. On the other hand *n* shows no tendency whatever to move forwards, but may do one of two things: it may remain where it is, or be shifted backwards only to a slight extent, in which case the hinder end of the body retains the sharp point characteristic of the trypanosome in the blood (Pl. 43, fig. 301); or it may pass back towards the extreme posterior end, and

even become terminal in position, in which case the hinder end becomes bluntly pointed or even rounded (Figs. 290, 291, 308). If at the same time the body becomes broadened out posteriorly, as sometimes happens, the result is a form which may mimic very exactly the small stumpy trypanosome which is the final form of the development (Figs. 299, 306, 307).

The trypanosomes that undergo this process of degeneration show a great tendency to adhere together in clumps attaching themselves to one another by the tips of their flagella (Pl. 43, figs. 304, 308; Pl. 44, fig. 311). The adherence in this way of the degenerative forms must be distinguished clearly from the process of agglomeration which *T. lewisi* undergoes so readily when placed in unfavourable circumstances.¹ Agglomeration takes place by the hinder ends of the trypanosomes and more especially by their *m*, as Laveran and Mesnil have shown, and as a result of it the trypanosomes tend to form rosette-like clusters, in which the flagella radiate outwards. True agglomeration of this kind can also occur in the flea under special circumstances, as will be described presently. But in the degenerative clusters the conditions are precisely the opposite to agglomeration, since the flagella are directed towards the centre of the cluster, while the hinder ends of the trypanosomes radiate outwards. The tendency of the degenerative forms to adhere in clumps must be interpreted as an expression of the general tendency (perhaps it might be termed instinct) of the trypanosome to attach itself by the tip of the flagellum to firm surfaces when in the body of the flea, a tendency very pronounced in all developmental forms, excluding the final stage of the cycle.

Clumps and masses of very considerable size are formed by the degenerative forms adhering together in the manner described. Towards the centre the clumps often show a cement-like substance, which stains pinkish-red with Giemsa. The final stages of the degeneration are small forms, which

¹ Manteuffel (1909) has already drawn attention to the distinction between rosettes, with flagella directed inwards, and true agglomeration.

represent simply the hinder ends of the original trypanosomes. They are usually sharply or bluntly pointed (Pl. 43, figs. 302-304), or may be rounded off (fig. 306). The large clumps of these little degenerative forms in the rectum are often very difficult to distinguish in the living state from the clumps of developmental crithidias. The degenerative clumps, however, generally occur loose in the cavity, while the true crithidias are attached to the wall of the rectum, though in the process of dissection the latter often become torn away from the wall. When a loose clump of this kind consists entirely of forms with pointed hinder ends it is probably degenerative. The true crithidial clumps always have a considerable number of forms with rounded hinder ends, especially in the early periods of the establishment of the rectal phase, when the hinder ends of the crithidial forms are almost always rounded. The degenerative forms, carefully examined, show a certain extent of undulating membrane running down the side of the body to *n*, which is situated behind *N*, while in the typical haptomonad phase the flagellum is reduced to the rhizoplast which comes off close to *n* and terminates at the surface of the body, *n* being situated beside or in front of *N*. Finally, it should be noted that the degenerative forms never multiply by division at any time. Nevertheless, in spite of all these distinctions, which are more easily perceived in permanent preparations than in the living state, it is sometimes difficult to pronounce decisively as to the nature of a given individual, whether degenerative or developmental, in preparations of the rectum; but as a rule there is no difficulty at all.

A remarkable fact is the occurrence of recurved forms amongst the degenerative forms in the rectum, of a type essentially similar to the recurved trypanosomes occurring in the normal developmental series in the stomach (Pl. 43, figs. 297, 298). The recurved forms are often seen in the clumps of degenerative trypanosomes. The occurrence of such forms in the rectum may perhaps be interpreted as an abortive effort on the part of the trypanosomes that have

passed on prematurely into the rectum to go through a development similar to that which they undergo normally in the stomach, but which, in all probability, would be impossible in the rectum, where the cuticular lining would doubtless be an effective bar to the penetration of the epithelial cells by the trypanosome. It is possible that some of the recurved forms in the stomach may also degenerate without ever succeeding in penetrating the cells; the curious forms such as Pl. 43, fig. 305, are very probably to be explained as recurved forms in process of degeneration. It would be difficult, however, as a rule, to distinguish between degenerative and developmental trypanosomes in the recurved condition in the stomach; but in the rectum all such recurved forms must be regarded as abortive and destined to degeneration.

As has also been mentioned above, trypanosomes of degenerative type are found in the rectum on the third and fourth days after infection. Such forms may, in some cases, differ but little from ordinary blood-trypanosomes, and are then to be interpreted, probably, as trypanosomes ingested at a later feed, which have passed on to the rectum; but in other cases they may be forms which are undergoing degeneration after having developed normally in the stomach. They are found, not infrequently, mixed with true developmental forms in clumps, into which they have probably intruded themselves (Pl. 41, fig. 183). It is necessary to be careful not to confuse them with early forms of the rectal phase in which *n* is still behind *N*; such forms can be distinguished by their greater stoutness and bulk, and by the fact that *n* has generally migrated forwards to some extent (Pl. 41, figs. 181-187).

True agglomeration very rarely occurs in the flea, but we have found it in its most typical form (Pl. 43, figs. 309, 310) in fleas of a batch, the record of which was as follows: The fleas, twelve in number, had been fed once on an infected rat in the usual way, and three days later they were fed again on a rat, the object being to test the influence of a second feed of clean blood on the persistence of the stomach-phase

(see p. 664 below). By mistake, however, the fleas were fed again on an infected rat instead of a clean rat. The next day (four days after the first infective feed) the fleas were dissected and examined. In every flea the trypanosomes of the second feed could be recognised, quite unaltered from the blood-form, and in most cases agglomerating in pairs, threes, or rosettes composed of many individuals; they were found in the stomach in every flea and in a few in the rectum also, where in one case degenerative forms were noted; in some fleas these trypanosomes were very numerous; in others they were scanty and had evidently undergone reduction in number. The trypanosomes of the first feed had disappeared in nine out of the twelve fleas, while in the remaining three they were represented by developmental forms of the usual type in the rectum. Agglomerating trypanosomes of the second feed were found both in fleas in which those of the first feed had persisted and in fleas in which they had disappeared.

From this observation it would appear that when a flea has once had an infective feed, its digestive tract, and more especially its stomach, acquires properties which cause the agglomeration and probably also the degeneration of trypanosomes taken in at later feeds, alike whether those ingested at the first feed have succeeded in establishing themselves in the flea or not.

Reference has already been made above (pp. 531-532) to intracellular forms which appear to be undergoing degeneration after having penetrated into an epithelial cell (Pl. 36, figs. 43-45).

APPENDICES TO THE DEVELOPMENT.

(1) Previous Investigations on the Development of *Trypanosoma lewisi*.

The first who attempted to follow out the development of *T. lewisi* in its invertebrate host was Prowazek (1905), who studied the development in the rat-louse, *Hæmatopinus spinulosus*, and since those

who followed immediately after him in similar investigations also made use of the louse, it is simplest to deal first with all those works in which the development in the louse is studied. Since we have not ourselves studied the development of this insect, we are not in a position to controvert the statements made, but it is legitimate for us to compare the forms and stages described with those which we have found in the flea, and, on the ground of such comparisons, to criticise the interpretations given by the authors.

According to Prowazek, the general course of the development in the louse is as follows: The flagellates are first to be found in the stomach, where they do not collect at particular spots, but swim freely everywhere in the ingested blood. In the stomach the processes of maturation and fertilization take place. At the second feed of the louse the parasites are forced down to the end of the mid-gut and finally come to rest in the hind-gut, for the most part near the Malpighian tubules, but also in other parts. Resting stages are to be found on or between the cells of the mid-gut, and more especially at the beginning of the hind-gut. From the fact that the parasites disappear from the hind-gut, it is inferred that they can pass through the epithelium of the hind-gut. [This conclusion is by no means warranted by the observation on which it is founded; it is also, in our opinion, extremely improbable that the flagellates could penetrate through the chitinous cuticle lining the hind-gut.] In this way the parasites are stated to pass into the blood-stream, then into the larynx [sic], and so finally back into the vertebrate host when the louse feeds. [It is not clear whether this statement is founded on observation, or simply on the analogy of the statements made by Schaudinn with regard to *Trypanosoma noctuæ*; the author never succeeded in obtaining an infection of the rat by means of the louse.] No trypanosomes or their resting stages were found in freshly-deposited faeces.

The author describes in great detail, with figures, various appearances interpreted by him as maturation, fertilisation, and even parthenogenesis. As all this part of the work is in the highest degree unconvincing and appears to consist of forced theoretical interpretations of degenerating forms which were occasionally seen to undergo agglomeration, it is not necessary to do more than refer to the figures given by Prowazek. Pl. II, fig. 32, purports to show the "fertilisation" in the living, and Pl. III, figs. 38 and 39, in the stained condition, while fig. 40 represents the "ookinete," a non-flagellated form with a single nucleus. From the ookinete a crithidial form is stated to arise in the manner described by Schaudinn (Pl. III, figs. 41-43 and 45). An active multiplication follows [but Pl. III, fig. 55, which is given as an example of the division, is simply an unaltered blood-trypanosome which shows the commonly-occurring abnormality of possessing two *NN*; compare our Text-fig. 15].

The author appears to regard most of the crithidias as involution-forms, which form "agglomeration-stars." Such a star is shown in Pl. III fig. 54 [which represents a typical clump of normal developmental crithidias, similar to our Pl. 41, figs. 182-184]. In addition to the crithidial involution-forms there are found other much smaller forms, wedged in between the cells and with the flagella completely absorbed. The crithidial involution-forms may also degenerate into small non-flagellated forms (fig. 50). No special inoculative or final form of the development is described.

Baldrey (1909) professes to have confirmed the development described by Prowazek, including even the process of maturation and fertilisation.

TEXT-FIG. 15.



Trypanosome with two NN from the stomach of a flea eighteen hours after the infective feed. Such forms with two and even three NN are quite common in the blood of the rat and in the gut of the flea at early stages of the development; compare Minchin (1909), p. 803, Pl. 21, fig. 6, Pl. 22, fig. 74, and Pl. 23, fig. 84; they have nothing to do with reproduction of the trypanosome by fission. ($\times 2000$.)

He gives two text-figures, one showing "male" and "female" forms and "copulation," the other showing "ookinetes" and crithidial forms of the typical nectomonad type. He states that the ookinete reconstructs a flagellar apparatus and divides rapidly to produce crithidial forms, which, by repeated division become smaller and smaller, pass into the body-cavity, thence to the suctorial mouth-apparatus and so infect the rat. The complete cycle takes from eight to ten days.

Rodenwaldt (1909) studied the development of *T. lewisi* in the louse in order to meet the criticisms of Patton, and succeeded incidentally in proving that Patton's "*Crithidia hæmatopini*" is a mythical and non-existent species. On the first day of the development he found in the louse both unaltered forms of *T. lewisi* and long forms,

which he described as "Lanzettformen." [The latter, from the figures given (Pl. 1, figs. 7-12) are clearly the same as our long "crithidiomorphie" stomach-forms.] He also found trypanosomes alleged to be dividing (figs. 5, 6, and 12) [but these, again, are simply forms with two or three *NN*, such as occur frequently in the blood of the rat; see above]. On the second and third days he found the same forms, but a larger proportion of the Lanzettformen. In one louse, however, he found crithidial forms developed on the third day. On the fourth day he found forms with a short flagellum or none at all (as shown in his figs. 13, 14), which he compares with the ookinetes of Prowazek and Baldrey; they are stated to bend their bodies without changing their place. On the fifth day long crithidial forms appear (compare his figs. 15-21), and on the sixth and following days smaller crithidial forms in rosettes, and also, but more rarely, leptomonad forms (figs. 23, 27). From the tenth day there were found (1) small non-flagellated forms (figs. 31-34); (2) stout flagellated forms (figs. 35-39); (3) a few stout, non-flagellated forms, "ookinetes" (figs. 40-47); and also forms regarded as representing copulation of gametes (figs. 48, 49). After twenty days slender, sporozoite-like forms were found (figs. 52-58) in the gut, never in the body-cavity. Rodenwaldt did not succeed in producing infection by means of lice.

Breinl and Hindle (1909) describe the development in the louse mainly as follows. The ingested trypanosomes first show characteristic changes in the nucleus, of which the karyosome divides and the division products move to opposite ends of the nucleus. *N* and *n* then become approximated and a division takes place. Some of the trypanosomes show about this stage a reduction of the cytoplasm, producing tadpole-like forms with a swollen head and the rest of the body reduced to a long flagellum (see their figs. 5-9); at this stage the two nuclei take on the crithidial ("Herpetomonas-like") arrangement. The crithidiæ multiply by division and become "agglomerated in great clusters with the flagellum always directed inwardly" (fig. 18). [These clusters appear to be simply developmental clumps of crithidial forms; as pointed out above, clumps with the flagella directed inwards, whether of degenerative or developmental forms, are not instances of true agglomeration.] Round forms [haptomonads?] are also found (figs. 32-36). The alleged conjugation was not confirmed.

[We cannot help remarking that all the stages of the trypanosome figured by Breinl and Hindle, even the crithidial clumps, present an extraordinarily sickly and degenerative appearance; we venture to think that anyone who compares their figures with ours will agree to this statement.]

Swellengrebel and Strickland (1910), have had the advantage over previous authors that they were able to compare the stages in the louse

with those occurring in the flea; their memoir is illustrated by numerous figures, for which, however, they appear not to claim great exactness, since they refer to them as "diagrams." They find that "the development in the louse is a very irregular one and is not to be compared with that which takes place in the flea." The first changes are that *n* wanders in the direction of *N*, producing finally a crithidial form, slender or club-shaped (diagrams xv and xvi). From the crithidial forms arise large "ovals," some of them without flagella and representing the "ookinetes" of former authors. Later ovals [haptomonads] and crithidia [nectomonads] are found singly or in clumps (diagrams xvii and xviii). Degenerative forms were also seen, but no flagellates were found which could be identified with this small, final trypanosome-forms of the development in the flea. No conjugation was observed.

From all these various works, only one positive fact emerges clearly, namely, that *T. lewisi* can develop in the louse into its typical crithidial phase, with both nectomonads and haptomonads. On the other hand, no intracellular multiplication has been observed, nor has it been proved as yet that the development can proceed so far as to produce the small trypanosomes which end the cycle in the flea. One form, apparently degenerative, occurs in the louse which we have not found in the flea, namely, a large oval form without a flagellum, the zygote or "ookinete" of Prowazek and others.

The first published works on the development of *T. lewisi* in the flea were those of Swellengrebel and Strickland (1910). We have already noticed above their statements with regard to the development of the stomach-phase and stated that we are quite unable to agree with the account given by them. On the third day of the development they find both long crithidial forms and large "ovals" [stout crithidias of the haptomonad type] in the mid-gut (diagram v). On the fourth day they state that the flagellates had all passed out of the stomach into the intestine. On the fifth day they found only round forms [haptomonads] in the rectum (diagram vii). On subsequent days they found the various forms of the rectal phase, and on the eighth day they found the small trypanosome-forms, the final stage of the development, which the authors were the first to discover. In their diagram xvi. they give a summary of the development showing the following sequence of forms: (1) the normal blood-trypanosomes; (2) a long crithidial form; (3) a stumpy crithidial form with short flagellum; (4) a haptomonad form; (5) the same in process of division; (6) a form transitional to—; (7) a nectomonad; (8) a form transitional to—; (9) the final trypanosome-form. [In view, however, of the great differences seen in the development of the trypanosomes in different fleas, especially prior to the establishment of the rectal phase, it was somewhat rash to attempt to fix the order of events in so few as eighty-three fleas, and it may be

remarked that the entire stomach-phase has practically been omitted from the cycle as summarised by the authors.]

Swingle (1911) gave the following description of the cycle in the flea. He states that the trypanosomes remain but a short time in the stomach, but migrate to the intestine where important changes take place. The first change to be seen is a diminution in size, and at the same time *N* moves towards the posterior end of the body. Occasionally such forms degenerate; in those that do not *n* moves forwards till it is close beside or in front of *N*, thus producing a true crithidial form. The individuals which do not change into the crithidial type curl upon themselves to form an oval rounded mass (figs. 15, 16) [apparently representing recurved forms]. Development of the crithidial forms may proceed along two separate lines which come to the same end; (1) they may "agglutinate" by the anterior ends forming rosettes (figs. 20, 21 [representing typical early crithidial clumps]); or (2) they may form solitary cysts (figs. 22-30) [apparently representing typical examples of the degenerative series]. Other forms [apparently degenerative] are also described; but the haptomonad and other forms of the rectal phase are all referred by the author to the form-series of the leptomonad described by him as *Herpetomonas pattoni*; a conclusion which Swellengrebel and Strickland (1911-12), justly criticise, though they go too far in the opposite direction in suggesting that *H. pattoni* is a stage in the development of *T. lewisi*.

Nöller (1912), studying the development of *T. lewisi* in the dog-flea (*Ctenocephalus canis*), confirmed our discovery of the intracellular multiplication in the stomach and added some further details; he observed the penetration of a trypanosome into a cell five hours and fifty-five minutes after the ingested blood had been ingested by the flea and states that the trypanosomes go through at least two generations, probably more, of intracellular multiplication. Whether or not the trypanosomes establish a normal infection in the flea depends, in Nöller's opinion, upon whether they succeed in fixing themselves in the intestine or rectum, or not. As regards the multiplication of the attached forms in the end-gut, Nöller finds that they can always be distinguished from the leptomonads by the possession of a typical undulating membrane and by undergoing a process of multiple fission; neither of these statements accord in the least with our experience of the development of *T. lewisi* in *Ceratophyllus fasciatus*.

It remains to mention that Swellengrebel and Strickland (1910) made some observations on the development of *T. lewisi* in *Ornithodoros moubata* and *Cimex lectularius*. In the former they got no development of crithidial forms; in the latter they found large crithidias but no development in the hinder part of the mid-gut.

(2) On the Possibility of the Occurrence of Sexual Phenomena in *T. lewisi*.

It has been seen from the foregoing summary of previous investigations on the development of *T. lewisi* that Prowazek first, and after him Baldrey, Rodenwaldt, and Gonder, asserted that the development of *T. lewisi* in the louse begins with a process of fertilisation, of which the main features are stated to be as follows: Slender male and stout female forms of the trypanosome are differentiated; their nuclei go through a process of maturation and reduction, after which a fusion of the gametes takes place. The zygote is described as an "ookinete" of elongated, oval form, with no flagellar apparatus and with a single nucleus (synekaryon). The nucleus is then stated to divide into two by a heteropolar mitosis to produce the two nuclei of a trypanosome n and N , and then the locomotor apparatus, flagellum and undulating membrane, are formed. The result is a flagellate of crithidial structure, which proceeds to multiply actively by binary fission.

It must be remarked here that Prowazek's account of the "ookinete" and its development in *T. lewisi* was modelled in every essential detail on the account given by Schaudinn for *Trypanosoma noctuæ*. The fertilisation observed by Schaudinn, however, was not that of a trypanosome, but of *Hæmoproteus* (*Halteridium*). It is a process of true fertilisation, which was first observed *in vitro* by Macallum, and its occurrence is not open to doubt. Schaudinn differed from all previous investigators in asserting that the ookinete (zygote) of *Hæmoproteus* became converted into a crithidial flagellate, a statement which has never been confirmed, and seems never likely to be. There can be little doubt at the present time that in linking together the development of *Hæmoproteus noctuæ* and of *Trypanosoma noctuæ* into a single life-cycle Schaudinn fell into error. Prowazek, on the other hand, derived his "ookinete" in *T. lewisi* from the sexual union and fusion of two trypanosomes, so that in its alleged origin the ookinete of *T. lewisi* is of quite different nature from that of *Hæmoproteus noctuæ*. Prowazek is, therefore, the first investigator who claims to have seen sexual conjugation of trypanosomes in the invertebrate host.

Later investigators of the development of *T. lewisi* in the louse have not confirmed Prowazek's statements as regards the sexual phase, nor has anything similar been found in the flea. Those who have investigated the development of other trypanosomes in their invertebrate hosts have also failed altogether to observe sexual phases or sexual behaviour, in spite of much careful searching for phenomena to which their attention has been strongly directed. As stated above, Prowazek's account of the sexual processes is most unconvincing, and the data he

brings forward are quite inadequate to support the superstructure of theoretical interpretation built upon them. In short, the question of sexuality in trypanosomes may be summed up in the words of Miss Robertson (1912, p. 247): "There is at present no sound evidence of conjugation in any trypanosome life-cycle so far worked out."

We have ourselves searched most carefully, but in vain, for sexual phases and syngamy in the development of *T. lewisi*. As stated above (p. 519), we found in one flea long crithidial forms adhering in couples in a manner very suggestive of true sexual behaviour, and believed that we had observed true syngamy. We were never able, however, to confirm this observation or carry it any further, and we are now convinced that the phenomena observed on that occasion were simply processes of agglomeration of abnormal forms of the trypanosomes in a malformed flea. When we discovered the stomach-phase we thought it very probable that the sexual processes might take place in this part of the developmental cycle, and we were inclined to interpret as evidence of sexual union some of those stages with two *mn* and two *NN*, such as Pl. 36, figs. 19-23, which are certainly at first sight very suggestive of the fusion of two trypanosomes. We have no evidence, however, of any subsequent fusion of the nuclei, nor of any antecedent processes of nuclear reduction such as should be the preliminary to the process of syngamy. We are not able to arrange the figures of these stages in any series which would suggest a sexual process. In short we are not able to interpret these forms as anything but early stages of the multiplication of the trypanosome.

On the other hand, it has been shown convincingly that the cycle in the invertebrate host effects a marked change in the properties or idiosyncrasies of the trypanosomes that have undergone it. Gonder showed that an arsenic-resistant strain of *T. lewisi* remained arsenic-resistant so long as it was transmitted from rat to rat by direct inoculation, but lost that property when transmitted by the louse. Miss Robertson (1912) also found that strains of *T. gambiense* became changed in character when transmitted through the tsetse-fly, and remarks: "It seems clear that the cycle in the fly as a whole, whether conjugation actually occurs or not, has much of the biological significance of the process."

Those who believe that trypanosomes pass through sexual phases in their invertebrate host will be inclined to ascribe the changes in the properties of the parasite to the effects of the sexual process. At the present time it is not possible either to affirm or to deny, with certainty, that sexual processes occur. All that can be said with any approach to verisimilitude is that the change appears to be connected in some way with the metamorphosis of the trypanosome and its passage through a crithidial stage; but proof is lacking that the crithidial stage follows

upon, and is the product of, a sexual process. Attention may be drawn here to another possibility already indicated above, namely, that the crithidial phase may be initiated by a differentiating division into two inequipotential products, one of which is destined to be eliminated sooner or later from the direct line of the life-cycle. If this supposition is correct a possible explanation might be afforded for the renovating effects of the invertebrate cycle. In the present state of knowledge, however, such an explanation must remain hypothetical, and lacking objective foundation.

PART III.—EXPERIMENTAL STUDY OF THE PROBLEMS OF THE TRANSMISSION AND DEVELOPMENT.

(1) INTRODUCTION.

THROUGHOUT our investigation of the relations of *Trypanosoma lewisi* to the flea, we have endeavoured, as far as possible, to make experiment and observation go hand in hand, employing the one method to check or throw light upon the results obtained by the other. In the following pages we set forth our results in a number of sections which arrange themselves naturally into two groups. One group (sections i–xv) embraces problems that deal with the complete cycle (including the passage of the propagative forms back into the rat) and with the establishment of *T. lewisi* in the flea, raising questions that are of general interest in the study of trypanosomiasis. The other group (sections xvi–xix) deals with some further problems that are of interest more especially in relation to the flea *Ceratophyllus fasciatus* and to *T. lewisi* itself under more or less special conditions in the flea. Each section is headed by a proposition which it is the object of the experiments cited to establish. If we consider that the proposition is proved satisfactorily by our experiments, it is put in the form of a positive or negative statement; if, on the other hand, the problem stands in need of further proof, the heading of the section is expressed in interrogative form.

The details of each experiment are given when it is cited,

but a few general remarks upon our methods may be made conveniently at this point. We kept going two breeding-cages of the type used by the Plague Commission (see 'Journal of Hygiene,' vi, Pl. iv). In one cage a clean rat was always kept to feed the fleas, in the other an infected rat; these two cages are designated, in the account of our experiments, the non-infected and the infected breeding-cage respectively. From the former we could always obtain a plentiful stock of clean fleas when required, while the latter furnished infective fleas. The rats used were almost always white rats bred in captivity; we found them as a rule docile and good-tempered so long as they were handled with the hands and not with forceps, and the operation of pricking their tails to obtain drops of blood, when required, did not arouse their resentment in the slightest. They live well in captivity, and were none the worse for being exposed to the fleas in the breeding-cages, provided the number of fleas was not allowed to become too great. Many of them suffered, however, from a troublesome itch, caused by a minute Acarine, which is very difficult to get rid of. One of our breeding-cages became over-run by rat-mites, rendering it necessary to destroy it and start a fresh one.

For our actual experiments we used in many cases, especially for experiments with small numbers of fleas, cages of special design in the form of a cylindrical tin-canister with the bottom closed in with tin, the top provided with a lid with a tightly-fitting rim. The canister was 10 in. high and 6 in. in diameter. The top of the lid was made of strong wire gauze, to prevent the rat jumping out, and over that muslin-gauze was pasted to prevent escape of fleas. After these cages had been in use for some time, however, they tended to become rusty on the inside and then the fleas could climb up the tin easily. Consequently, it was found more suitable to use inverted bell-jars, each about $15\frac{1}{2}$ in. in height and 7 in. in diameter. The bell-jars were supported each on a wooden block, or several together in a wooden crate. The open upper end of the bell-jar had a zinc wire cover to

prevent the rat jumping out, but it was not necessary to take precautions against the fleas escaping, because they are unable either to jump so high or to crawl up the smooth glass if kept clean. The bell-jars were cleaned out thoroughly once a week.

The bell-jars were especially suited for experiments with single fleas or a small number of fleas. First of all clean saw-dust is put in the bell-jar to a depth of about 3 in., then the rat and the flea or fleas are put in. Since *Ceratophyllus fasciatus* is a flea which does not live permanently on the rat but only goes on to it for food, and lives naturally in rat-burrows, the fleas were generally to be found without difficulty in the saw-dust, when it was necessary to recover them, but sometimes they were on the rat itself. In the latter case the rat was held over a deep bowl of enamelled iron and the flea disturbed by blowing on to the fur of the rat, which has the effect of soon making the flea come to the surface of the fur. It was then captured, as a rule, by seizing it gently by finger and thumb, an operation which must be performed rapidly and deftly, otherwise it burrows down into the fur and must be dislodged again. Sometimes the flea drops off the rat and falls into the bowl, where it can be recaptured easily. Our assistant, Mr. George Kauffmann, became exceedingly expert at this job, and if a flea could not be found by him it was safe to assume that it had died or been eaten. In our earlier experiments we used chloroform for recovering the fleas, but later we abandoned this method, often fatal to the rats.

In some cases it was required to expose a large number of fleas—200 or so—to infection on an infected rat for a night or a day. For this purpose the bell-jar was also handy, but it was often found that the rat ate a great many of the fleas, sometimes as many as 100 or more in a single night. To prevent this a cylinder of wire gauze was made, of sufficient length to fit into the bell-jar in such a way that its two ends were closed by the glass wall of the jar, and of such a calibre as to allow the rat to walk forwards or backwards along it,

but not wide enough to permit the rat to turn round or use its paws freely, and consequently hindering it from catching and eating the fleas.

For the purpose of collecting large numbers of fleas from the breeding-cage the following method was found to be the simplest: Two glass capsules were used, each provided with a well-fitting lid, the one smaller, about $2\frac{1}{4}$ in. in diameter and $1\frac{1}{4}$ in. in height; the other larger, about 6 in. in diameter and 3 in. in height. First of all, débris from the breeding-cage containing fleas in all stages of their development is scooped up with the small capsule and the lid at once clapped on. Then the small capsule is placed in the large one; the lid of the small capsule is removed with one hand, and the lid of the large one put on with the other. The adult fleas in the small capsule then begin at once to jump out of it in every direction, and so fall into the enclosing large capsule, in which they soon collect on the side furthest from the light. When the fleas have swarmed out in this way the small capsule is removed and the débris contained in it is returned to the breeding-cage. The fleas in the large capsule can then be emptied through a glass funnel into a suitable receptacle, such as an Erlenmeyer flask. Or, if the large capsule be left to stand until all the fleas have congregated on the side furthest from the light, then by suddenly turning the capsule round through about 180° , so that the side which was furthest from the light is now the most illuminated, the fleas begin at once to move towards the opposite side; and if then the small capsule be held in their way they can be made to jump into it of their own accord, and they can thus very easily be counted and disposed of as required. *Ceratophyllus fasciatus* is not a very good jumper and its trajectory is low.

The fleas collected can be kept, if required, for a considerable time; we found the best method was to put a little clean white sand, moistened with two or three drops of water, at the bottom of a flask. The fleas burrow down into the sand and appear to live comfortably. If they are to be kept any length of time the sand must be moistened again every

two or three days. Like most blood-suckers, the flea can stand a prolonged fast.

(2) GENERAL PROBLEMS.

- (i) *Trypanosoma lewisi* is transmitted from Rat to Rat by the Rat-flea, *Ceratophyllus fasciatus*.

It is not necessary that we should cite experiments specially to prove this proposition, since it is established by the experiments brought forward under the headings that follow, and it has been proved beyond all possibility of reasonable doubt by experiments already published by others as well as by ourselves.

The agency of fleas in the transmission of *T. lewisi* was first demonstrated by Rabinowitsch and Kempner (1899), who succeeded in infecting clean rats by intra-peritoneal injection of teased-up fleas (species not stated) which had previously been fed on infected rats. In these experiments the trypanosomes appeared in the blood of the rats in six to eight days after the injection. The authors state that they were not able to find any stages of the trypanosome in the flea-débris which was injected. They also obtained positive results by placing fleas, previously fed on infected rats, upon clean rats; the trypanosomes made their appearance in the blood of the rats after two to three weeks. Their experiments with lice gave negative results.

In spite of the experiments of Rabinowitsch and Kempner, the work of Prowazek (1905) on the development of *T. lewisi* in the rat-louse, *Hæmatopinus spinulosus*, led to this insect being regarded as the true host of the rat-trypanosome, and no more experiments with fleas appear to have been undertaken until those of Nuttall (1908), who obtained positive infections of rats with fleas, using both *Ceratophyllus fasciatus* and *Ctenophthalmus agyrtes*. Two years later we published accounts of a number of experiments, since when the rôle of the flea has been established beyond the necessity of further experiment upon the subject.

We have confined our experiments throughout solely to the common English rat-flea, *Ceratophyllus fasciatus*, but it has been shown by Nöller (1912) and Wenyon (1913) that the transmission can be effected by other species of fleas, namely, the dog-flea, *Ctenocephalus canis*; the human flea, *Pulex irritans*; and the Indian rat-flea, *Xenopsylla cheopis*. It is indeed noteworthy that other species of fleas appear to be more efficient as true hosts of the rat-trypanosome than the species which in this country occurs habitually

in association with rats, since Dr. Wenyon has informed us that in the fleas with which he experimented, the trypanosomes never failed to establish themselves and to go through their complete developmental cycle, while in *Ceratophyllus fasciatus* we found that only a small percentage of the fleas became infective (see below), and examination of the fleas showed that the trypanosomes establish themselves in a correspondingly small percentage (see p. 659). It would appear, therefore, that the flea which, more than any other species, is exposed in this country to infection by *T. lewisi*, has developed a certain degree of natural immunity to the parasite. From the experiments published by the authors cited it is probable that *T. lewisi* would undergo its development in any species of flea, and would be transmitted by it, provided that the flea could be induced to suck the blood of an infected rat. The natural efficacy of any given species of flea in transmitting *T. lewisi* depends probably on the habits and tastes of the flea, and not on any specific ability to harbour the trypanosome. Brumpt (1913) has pointed out that all the trypanosomes of small rodents seem to be able to develop in fleas.

A number of experiments have been performed by several investigators on the transmission of *T. lewisi* by means of the rat-louse, *Hæmatopinus spinulosus*. The first experiment with rat-lice (species not stated) was carried out by MacNeal (1904), who transferred "several" lice from an infected to a clean rat; trypanosomes appeared in the latter after fourteen days. Positive results in experiments of this kind with rat-lice are reported by Nuttall (1908), Baldrey (1909), Breinl and Hindle (1909), Manteuffel (1909), and Gonder (1911). To judge, however, from the published accounts of these transmission-experiments, positive results were by no means frequent and were obtained in some cases at least with difficulty and by the exercise of great patience and perseverance, or by using large numbers of lice. Nuttall obtained one positive result in an experiment in which sixty lice were used; two other experiments, in which fewer lice were used, were negative. Baldrey reports two experiments in which infection was obtained by means of lice; in the first, 100 lice were used, and the result is regarded by him as a case of direct mechanical infection, but for what reason is not at all clear; the second, in which ten lice were used, is interpreted as demonstrating a developmental cycle in the louse. Breinl and Hindle report three successful transmissions by means of lice, after carrying on numerous experiments for over a year. Manteuffel seems to have been more successful than most other experimenters in this field, though he does not record the actual number of his experiments or the proportion of those which were positive in result, but he states that infections with lice were "prompt and frequent"; his method was to put infected rats, with lice on them, in the same cage with clean

rats, and from his results he concludes that lice do not transmit the trypanosome longer than from three to five days after being removed from the infected rat, and that the transmission is effected by the act of blood-sucking; if the first of these two conclusions be true, it would appear that the trypanosome does not succeed in establishing itself in the louse in the way it does in the flea. Gonder reports that after many fruitless attempts to transmit *T. lewisi* with definite numbers (80-100) of lice, he obtained six positive results in a series of fifty experiments, and eight positive results in another series of fifty, using greater numbers (grössere Mengen) of lice; and he also brought about six infections by making emulsions of lice taken directly from an infected rat, the lice having been left on the infected rat for five, nine, eleven, thirteen, sixteen, and twenty-one days respectively, in these six experiments. On the other hand, Prowazek, who first described developmental stages in the louse and claimed that this insect was the true host of *T. lewisi*, was unable to obtain experimental transmission; Rodenwaldt obtained no positive results with numerous transmission-experiments; and we also have obtained only negative results in any attempts that we have made to transmit *T. lewisi* by means of the rat-louse.

It is evident from the results summarised briefly in the foregoing paragraph that transmission of *T. lewisi* can be effected by the rat-louse, but only with difficulty, and in a small percentage of cases. This is a great contrast to the ease and comparative certainty with which the trypanosome can be transmitted by fleas. We have always used our flea-cages as the simplest and easiest method of obtaining infected rats when required by ourselves or by our colleagues or friends, and not only have we infected rats with single fleas on many occasions, but we have even succeeded in infecting several rats successively with one and the same flea. We have no hesitation, therefore, in regarding fleas as the usual agency whereby *T. lewisi* is transmitted from rat to rat in Nature, a result brought about by the louse rarely and exceptionally.

It should be noted that Brumpt (1913) has succeeded in infecting a rat with *T. lewisi* by inoculating it with the rectal contents of a bug, *Cimex lectularius*, fed on an infected rat thirty-eight and again six days previously. There is no evidence, however, that this insect transmits the infection naturally.

- (ii) The Transmission takes place by the Cyclical Method. Transmission by the Direct Method has not been proved to occur.

These are among the conclusions drawn from experiments described in full detail in our preliminary report (1910). It

is sufficient here to state that experiments "A" (20) and "B" (21) in our report were devised chiefly to separate "direct" from "cyclical" infection, a matter of primary importance at the time that these investigations were begun, and they show that in the individual cases cited (A_3 and B_3) transmission was effected when all possibility of the direct method was excluded. Experiments "C" (22) and "D" (23) multiply such cases many times, and show further that fleas once infective retain the infection so as to infect a series of rats without themselves being exposed to fresh infection. Since then a number of experiments have been carried out by us, many of which are enumerated under the different headings which follow, and the sum-total of these experiments not only supports the conclusions in our preliminary report, but establishes beyond doubt that the rat-flea is a true intermediate host, that it can transmit the infection to other clean rats only after the developmental cycle has been completed within itself; that, in short, the infection takes place by the cyclical method; and that there is no evidence whatever to show that the rat-flea is capable of carrying the infection from one rat to another by what is called the "direct" or "mechanical" method.

The term method in the phrase "method of transmission," if used without qualification, should include comprehensively all that happens in the transmission of infection from one vertebrate to another. In the transmission of trypanosomes the natural transmitting agent, when such is known, is a blood-sucking invertebrate of some kind. When the method is said to be "contaminative" or "inoculative," transmission is viewed from the side of the invertebrate in its relation to the vertebrate, and the problems involved are particular, that is to say such as deal with modifications due to special circumstances in those relationships, and are concerned at most with special groups of trypanosomiasis rather than with trypanosomiasis in general. On the other hand when the method of transmission is said to be "cyclical" or "direct," transmission is viewed from the side of the trypanosome in its relation to the invertebrate, and the problem becomes a general one, dealing with that phase of the transmission which is concerned with the life-history of trypanosomes as a group of parasites, and with the wider question of their double relationship to vertebrate and invertebrate,

bringing them into line with other known relationships among parasitic Protozoa in this respect.

A great impetus was given to the study of trypanosomes by economic and other considerations arising out of the prevalence of tsetse-fly disease and sleeping sickness in Africa. It was long known that these diseases could be transferred artificially by direct inoculation of blood from a diseased to a healthy subject by means of a hypodermic syringe. Naturally, therefore, before much work had been done in this direction tsetse-flies known to be associated with the spread of these diseases were supposed to transmit them in this direct way. Bruce and others, experimenting with bred-out flies, proved the possibility of this taking place under certain conditions which, in the case of sleeping sickness at all events, were very unlikely ever to be fulfilled in Nature. Only with a swarming infection and by interrupted feeding could the disease be passed on directly from an infected to a clean animal with any approach to certainty, and even under the most favourable conditions in other respects, the longer the interval between interrupting the feed on the infected animal and continuing it on a clean animal, the less the chance of the clean animal becoming infected, until, with the lapse of about half-an-hour, it was just as certain that the infection would not take place. Moreover, however short the interruption between the feeds, an interposed partial feed on a clean animal rendered the fly non-infective to a second clean animal. Later experiments showed that the contents of the stomachs of flies that had fed on an infected animal, if injected into a clean animal, could produce infection only up to about two days after the infective feed. The fly itself, however, could not be shown to act in any way resembling a hypodermic syringe, and the idea of "delayed mechanical transmission" never found support from feeding experiments. The conclusion to be drawn from all the earlier experiments on direct transmission seemed to be that when infection was obtained it was with "fouled proboscis" before the blood in its lumen distal to the entrance of the salivary duct, and perhaps also on its external surface, had had time to dry, and that the conditions under which it was shown to be possible were never likely to be fulfilled in Nature, in the case of sleeping sickness, and in the case of tsetse-fly disease of cattle, far too seldom to account for the spread of the disease, while in no case could such a method of transmission account for the existence of fly-belts through which healthy domestic stock cannot pass. Other things being equal, the efficiency of an invertebrate as a transmitter of trypanosomes would be enormously increased if the invertebrate were a true intermediate host and not merely a "porter" of the parasites from an infected to a clean subject, and to demonstrate beyond doubt that trypanosomes underwent an alternation of

generations was of primary importance in connection with the general trypanosome problem at the time that we undertook this investigation, when it was being maintained by Patton and others that no trypanosomes went through a developmental cycle in the invertebrate, that all transmission of trypanosomes was direct, and that the crithidial forms found in blood-sucking invertebrates were all of them independent parasites of the invertebrate, having no connection with the trypanosomes or other parasites of the vertebrate. It was known that rat-fleas could transmit *T. lewisi* from infected to clean rats, and although transmission by fouled proboscides seemed quite out of the question, it was necessary to demonstrate beyond doubt that the rat-flea is a true intermediate host of *T. lewisi*, that it can transmit the infection to other rats only after the developmental cycle has been completed within itself, and that once infected it remains infective for a considerable time, so as to be able to infect a series of clean rats without itself being exposed again to infection. These points, which we believe concern the transmission of trypanosomes in general, and which may be taken as typical of the relations which trypanosomes as a group bear to their invertebrate hosts, as well as other points of more special interest (confined, it may be, to *T. lewisi* alone or to the *lewisi* group), are dealt with under different headings in what follows later. Although the practical cannot properly be separated from the scientific, the most interesting problem of the transmission from the scientific point of view is perhaps the way in which the trypanosome becomes established. There are considerable variations in the details of the cycles of different species or groups of trypanosomes in their natural hosts due to special conditions, but arising out of the very meaning of a cycle, and therefore common to all is the fact that until the cycle is completed the invertebrate, though infected, is not infective. This may be of direct practical importance in special cases, and where that is so it is important to ascertain the length of time required for the completion of the cycle in each case. Of more general practical importance in questions connected with the spread of infection is the fact that the trypanosome does establish itself in such a way that the invertebrate remains infective for a long time without requiring to be exposed again to infection.

- (iii) The Trypanosomes make their Appearance in the Blood of the Rat Five to Seven Days after Infection; the Multiplication of the Trypanosomes in the Blood of the Rat come to an End Eleven to Thirteen Days after Infection.

In order to establish with exactness the length of the

incubation-period and the multiplication-period in the rat after infection, it is necessary that the rat should have been exposed to infection by the fleas for a short time; long exposure leaves too wide a margin between the possible maximum and minimum deducible from the actual data furnished by the experiment for the mean to be of any value in reckoning the length of the two periods in question. When the rat is removed from contact with the infected fleas it is further very necessary that all fleas should be removed from its skin. The rat is then kept in a flea-proof cage and its blood is examined daily in fresh, living films until trypanosomes are first detected in it, in order to determine the duration of the incubation-period; then smears of the blood are made and preserved daily and examined until the multiplication-period is found to be past and ended. So long as the trypanosomes are multiplying in the rat's blood, they are of various sizes, some of the ordinary, normal size, others very small, and others again much above the normal size. Marked variation in the size of the trypanosomes is a sure sign that multiplication is proceeding, even when actual division-stages are so scarce in the preparation that prolonged search is necessary in order to find them. As soon as the multiplication is ended the trypanosomes are all of one type and size, allowing for slight individual variations that are not perceptible without careful measurement; to such trypanosomes, the normal form of *T. lewisi* and the sole form occurring in the blood when once the multiplication is at end, we shall refer always as "ordinary."

We cite here a few examples from our series of experiments, choosing first (Table C), those in which the rats were exposed to infection for one day only, so that the periods of incubation and multiplication can be determined within a margin of one day. In our second table (D), we quote those instances in which the rats were exposed to infection for two days, so that a wider margin of possible error must be allowed for in calculating the two periods. In a third table (E), we shall give some results obtained with rats which were infected

TABLE C.—One Day's Exposure to Infection.

No. of experiment.	No. of rat.	Put in.	Taken out.	Trypanosomes first seen.	Multiplication ended.	Incubation period.	Multiplication period.
20 (= A)	122	7: xii: '09	8: xii: '09	14: xii: '09	18: xii: '09	6-7 days	10-11 days
21 (= B)	123	7: xii: '09	8: xii: '09	14: xii: '09	18: xii: '09	6-7 "	10-11 "
22 (= C)	129	10: xii: '09	11: xii: '09	16: xii: '09	23: xii: '09	5-6 "	12-13 "
23 (= D)	138	16: xii: '09	17: xii: '09	21: xii: '09	Rat died	4-5 "	—
40	317	15: v: '11	16: v: '11	21: v: '11	26: v: '11	5-6 "	10-11 days
45	371	28: vii: '13	29: vii: '13	3: viii: '13	9: viii: '13	5-6 "	11-12 "

TABLE D.—Two Days' Exposure to Infection.

No. of experiment.	No. of rat.	Put in.	Taken out.	Trypanosomes first seen.	Multiplication ended.	Incubation period.	Multiplication period.
20 (= A)	130	11: xii: '09	13: xii: '09	17: xii: '09	22: xii: '09	4-6 days	9-11 days
21 (= C)	121	6: xii: '09	8: xii: '09	13: xii: '09	19: xii: '09	5-7 "	11-13 "
"	124	8: xii: '09	10: xii: '09	14: xii: '09	21: xii: '09	4-6 "	11-13 "
"	132	11: xii: '09	13: xii: '09	17: xii: '09	24: xii: '09	4-6 "	11-13 "
"	135	13: xii: '09	15: xii: '09	20: xii: '09	26: xii: '09	5-7 "	11-13 "
"	141	20: xii: '09	22: xii: '09	28: xii: '09	1: i: '10	6-8 "	10-12 "
23 (= D)	164	13: i: '10	15: i: '10	21: i: '10	26: i: '10	6-8 "	11-13 "
"	168	18: i: '10	20: i: '10	27: i: '10	30: i: '10	7-9 "	10-12 "
24	153	6: i: '10	8: i: '10	14: i: '10	18: i: '10	6-8 "	10-12 "
"	156	8: i: '10	10: i: '10	15: i: '10	21: i: '10	5-7 "	11-13 "

TABLE E.—Rats Infected by a Single Flea.

No. of rat.	Flea put on rat.	Flea recovered.	Trypanosomes first seen.	Multiplication ended.	Incubation period.	Multiplication period.
207*	2 : viii : '10	5 : viii : '10	11 : viii : '10	13 : viii : '10	6-9 days	8-11 days
212*	19 : ix : '10	22 : ix : '10	28 : ix : '10	30 : ix : '10	6-9 "	8-11 "
223*	10 : x : '10	14 : x : '10	17 : x : '10	22 : x : '10	3-7 "	8-12 "
233	20 : x : '10	25 : x : '10	28 : x : '10	31 : x : '10	3-8 "	6-11 "
244	11 : xi : '10	15 : xi : '10	23 : xi : '10	26 : xi : '10	8-12 "	11-15 "
255*	27 : iii : '11	1 : iv : '11	5 : iv : '11	7 : iv : '11	4-9 "	6-11 "
259*	23 : iii : '11	27 : iii : '11	30 : iii : '11	4 : iv : '11	3-7 "	8-12 "
263*	20 : iv : '11	25 : iv : '11	1 : v : '11	5 : v : '11	6-10 "	10-15 "
272*	14 : ii : '11	18 : ii : '11	24 : ii : '11	27 : ii : '11	6-10 "	9-13 "
291*	27 : iii : '11	1 : iv : '11	5 : iv : '11	9 : iv : '11	4-9 "	8-13 "
292*	25 : iv : '11	29 : iv : '11	1 : v : '11	6 : v : '11	2-6 "	7-11 "
296*	15 : iv : '11	20 : iv : '11	22 : iv : '11	25 : iv : '11	2-7 "	5-10 "
296*	17 : iii : '11	22 : iii : '11	27 : iii : '11	31 : iii : '11	5-10 "	9-14 "
315*	29 : viii : '11	left on	12 : ix : '11	16 : ix : '11	14 days (max.)	18 days (max.)
361*	13 : vii : '12	"	23 : vii : '12	26 : vii : '12	10 "	13 "
363*	13 : vii : '12	"	23 : vii : '12	26 : vii : '12	10 "	13 "

* The rats marked thus were examined only on alternate days. Consequently the dates of the appearance of trypanosomes in their blood, or of the termination of the multiplication period, may have been a day earlier than stated in the table.

TABLE F.—Rats Infected by Injection of Fleas.

Experiment.	No. of rat.	Date of injection.	No. of fleas.*	Trypanosomes first seen.	Multiplication counted.	Incubation period.	Multiplication period.
39	309	29: iv: '11	10 (r)	8: v: '11	10: v: '11	9 days	11 days
39	312	1: v: '11	10 (s)	8: v: '11	13: v: '11	7 "	12 "
39	314	4: v: '11	10 (s)	10: v: '11	15: v: '11	7 "	11 "
34	257	15: xii: '10	6 (s)	21: ii: '10	26: ii: '10	6 "	11 "
34	261	26: i: '11	6 (s)	1: ii: '11	6: ii: '11	6 "	11 "
32	282	14: ii: '11	1 (s)	20: ii: '11	(not noted)	6 "	—
32	287	14: ii: '11	1 (s)	20: ii: '11	"	6 "	—

* r = rectum; s = stomach.

each by a single flea, in order to show that in many cases, at least, the maximum periods of incubation and multiplication which can be deduced from experiments under these conditions are not greater than those indicated by the experiments in which many fleas were used to obtain infection. In a fourth table (F) we give for comparison, the results obtained by inoculating rats with the stomachs or recta of fleas; in such cases the length of the periods of incubation and multiplication can be determined with exactness, the moment of infection being known.

The determination of the length of the multiplication-period in an infected rat is of practical importance for interpreting other experiments, since, when it has been determined, it furnishes a datum from which approximately accurate conclusions can be drawn as to the time at which the rats become infected, when the point is shown definitely by the details of the experiment. As regards the first appearance of the trypanosomes in the blood, they appear at first in such scanty numbers that it is very easy to overlook them, and they may often be reported absent when a more prolonged search would have detected their presence. Similarly, the trypanosomes at the end of the multiplication-period may sometimes have been reported as "all ordinary" in a smear in which more careful searching might have led to the discovery of a few individuals above or below the normal size. Consequently, the errors of observation are such as tend to over-estimate the length of the incubation-period, and to under-estimate that of the multiplication-period, from the scrutiny of the blood-films. On the whole, however, the results obtained in our experiments are very uniform and indicate an incubation-period of about six days, a multiplication-period of about twelve days. It is interesting to note that these results agree with those obtained in the case of rats infected artificially by inoculation, intra-peritoneal or otherwise, with blood from an infected rat. Since a syringe would inoculate far more trypanosomes than the rat would obtain from even a large number of fleas, it might have been expected that the rat would, so to speak, fill up quicker when infected by means of a syringe, and that consequently the multiplication-period would be correspondingly shorter. In our experience, however, the length of the multiplication-period remains approximately constant in all cases, whether the infection is effected by a syringe, by a large number of fleas, by a few fleas, or even by a single flea; a fact which indicates that the length of time during which the trypanosome multiplies in the rat

is not determined by the number of trypanosomes put into the rat, but by the mutual interaction of host and parasite.

It may be noted here that some rats appear to possess a certain degree of natural immunity to infection with *T. lewisi*. A single instance which came under our experience will suffice to demonstrate this point. A rat was exposed to infection on June 9th and its blood was examined daily; on June 25th a few trypanosomes were first seen in the blood in scanty numbers, just as they are usually seen at their first appearance between the fifth and seventh days of the infection. The rat was then removed from contact with the fleas and kept apart; but neither on the next day nor on any subsequent day were any trypanosomes to be found in its blood. This rat, therefore, contracted only a transitory infection which was late in its appearance and disappeared after one day; had the trypanosomes been overlooked on that day the experiment would have been returned wrongly as negative in result.

(iv) The Cycle of Development in the Flea requires a Minimum of Five Days for its Completion.

This point was dealt with in our preliminary communication (1910), in which we came to the conclusion that the incubation in the flea was six or seven days. Our method of determining this was, first of all to expose non-infected fleas to infection, by putting them on a well-infected rat, for but a single day, so that if the fleas afterwards produced an infection, the time at which they themselves became infected could be determined within a narrow margin, twenty-two hours in our actual experiment. The fleas were then placed in contact for three days with clean rat (1) which did not become infected; after that for three days with clean rat (2), which also did not become infected; and then for two days on clean rat (3), which showed trypanosomes in its blood six days after being removed from contact with the infected fleas. Clean rat (3) was, therefore, infected by the fleas in the interval between the sixth and eighth day after the fleas themselves had acquired the infection; consequently the infection in the fleas could not have been more than eight days old.

Subsequent experiments performed by us have indicated a possible minimum of five days for the flea-cycle of the try-

panosome. In experiment 39 (see below, p. 630) it is proved that the rectum of the flea, injected into the rat, can produce an infection as early as the fifth day, and in such fleas the examination of films shows the presence of the small trypanosomes which are the final form of the development in the flea. In experiments 26 and 28, undertaken in order to ascertain whether a rat, in which the trypanosomes are still in the multiplication-period, is capable of infecting fleas (see below, p. 657), the results obtained indicated a short incubation-period in the fleas. Thus in experiment 26, 127 fleas, after being three days (from 8 : ii : '10 to 11 : ii : '10) on the infected rat were put on rat 187 for another three days (from 11 : ii to 14 : ii). Rat 187 showed trypanosomes in its blood after five days (19 : ii), and the multiplication-period ended five days later (24 : ii). Consequently the incubation-period in the fleas could not have been more than six days (8 : ii to 14 : ii). In experiment 28, 137 fleas were put first on the infected rat for four days (15 : ix : '10 to 19 : ix : '10), and then were put for one day (19 : ix to 20 : ix) on rat 209; after this they were put on rat 215 and left on it. Rat 209 had shown no infection when it died nine days later (29 : ix); rat 215 first showed trypanosomes on 30 : ix, and the multiplication was ended 3 : x. This result indicates that rat 215 was infected about 21 : ix, in which case the incubation-period in the flea could not have been more than six days (15 : ix to 21 : ix). Since rat 209 showed no trypanosomes for at least nine days after being exposed to infection it was probably not infected; so that the infection in the fleas was probably not ripe for at least five days (15 : ix to 20 : ix).

On the other hand we have instances, as already mentioned in our preliminary communication (1910), of an incubation-period in the flea apparently much longer than six days. Thus in experiment 19 a cage (J) was stocked with seventy-two fleas from the non-infected breeding-cage and an infected rat (No. 81, a wild black rat, naturally infected) was put with them for three days (20 : ix : '09 to 23 : ix : '09). Rat 81 was then removed and rat 82, a clean, tame rat, was put in its place (23 : ix) and left in the cage. Rat 82 first showed trypanosomes in its blood 29 : x; the multiplication-period was ended about 4 : xi, indi-

cating that the actual infection of rat 82 took place about 23: x. In this case, therefore, the fleas did not produce an infection in the clean rat for at least a calendar month after their contact with the infected rat was interrupted. Such a result, however, permits of no conclusion whatever as to the length of the incubation-period in the flea; it merely demonstrates a point proved also by other experiments, namely that infective fleas often fail to infect. We have put forward already (1910) one possible explanation for this, that a rat, which is comparatively immune to begin with, may resist infection for a long time, but its resistance may be overcome at last. Another possible explanation may be given by the method in which infection of the rat by the flea is now known to take place, namely by the rat licking off the moist faeces of infective fleas that are deposited on its skin (see below, p. 648). It is evident that if the rat fails to lick off the faeces while still moist, or if the infective flea does not defæcate on the rat, no infection is brought about. A negative result of this kind is most likely to be attained when the number of infective fleas on the rat is very small, as seen in the large number of negative and small number of positive results in our series of experiments in which single fleas were used (see below, p. 661). That infective fleas in Cage J were rare is shown by the fact that between 23: ix and 19: x thirty fleas from this cage were dissected and examined without finding a single one infected. On the other hand, when fleas are sufficiently numerous and have been well infected, positive results are fairly certain (Experiment "C" of our preliminary report).

- (v) Transmission is never effected until the Developmental Cycle is completed; that is to say, until at least Five Days have elapsed since the First Exposure of the Fleas to Infection.

We have found, as already stated, by direct observation, that the final form of the developmental cycle appears in the gut of the flea five days after the infective feed (see below, p. 630). We bring forward here a few instances to show that at least five days must elapse before the flea becomes infective, after having ingested trypanosomes from an infected rat.

(1) Experiment 20.—A cage colonised with forty-four fleas that had been exposed to infection from 4: x : '09 to 8: x : '09.

Rat 93 put into the cage from 8: x to 12: x, i. e. during a period in which the age of the infection in the fleas could not have been less

than two days old at the beginning nor more than eight days old at the end. Result negative.

(The next rat used in this experiment died, but subsequent rats used showed that the fleas had become infective.)

(2) Experiment 21.—A cage colonised with 157 fleas that had been exposed to infection from 11 : x : '09 to 15 : x : '09.

Rat 97 put in from 15 : x to 19 : x, i. e. during a period in which the age of the infection in the fleas could not have been less than a few hours at the beginning nor more than eight days at the end. Result negative.

(The next rat put in became infected, apparently about 27 : x; age of infection in the fleas then between twelve and sixteen days.)

(3) Experiment 22.—A cage colonised with 160 fleas exposed to infection from 24 : xi : '09 to 27 : xi : '09.

Rat 116 put in from 27 : xi to 30 : xi, i. e. during a period in which the age of the infection in the fleas could not have been less than a few hours at the beginning nor more than six days at the end. Result negative.

(The next rat put in became infected, apparently about 3 : xii; infection of the fleas then six to nine days old.)

(4) Experiment 23.—A cage colonised with 162 fleas exposed to infection from 7 : xii : '09 to 8 : xii : '09.

Rat 125 put in from 8 : xii to 11 : xii, i. e. during a period in which the age of the infection in the fleas could not have been less than a few hours at the beginning nor more than four days at the end. Result negative.

Rat 133 put in from 11 : xii to 13 : xii, i. e. during a period in which the age of the infection in the fleas could not have been less than four days at the beginning nor more than six days at the end. Result negative.

(The next rat put in became infected, apparently about 15 : xii; infection of the fleas then seven to eight days old.)

(5) Experiment 24.—A cage colonised with fifty fleas exposed to infection from 7 : xii : '09 to 8 : xii : '09.

Rat 126 put in from 8 : xii to 11 : xii, i. e. during a period in which the age of the infection in the fleas could not have been less than a few hours at the beginning nor more than four days at the end. Result negative.

(The next rat put in became infected, apparently about 3 : i : '10; age of the infection of the fleas then between twenty-six and twenty-seven days.)

(6) Experiment 25.—A cage colonised with seventy fleas exposed to infection from 31 : xii : '09 to 3 : i : '10.

Rat 152 put in from 3 : i to 6 : i, i. e. during a period in which the

age of the infection in the fleas was not less than a few hours at the beginning nor more than six days at the end. Result negative.

(The next rat put in became infected, apparently about 6 or 7 : i; the age of the infection at 7 : i was from four to seven days.)

(7) Experiment 45. Batch C (see below).—Bell-jar colonised with thirty fleas exposed to infection from 22 : vii : '13 to 23 : vii : '13.

No infection produced in rat 370 put in for a period of two days, during which the infection in the fleas could not have been less than five days old at the beginning nor more than seven days old at the end.

No infection produced in rat 370a, put in for a period of one day, during which the infection in the fleas could not have been less than eight days old at the beginning nor more than ten days old at the end.

(The next rat put in became infected.)

In the previous section it has also been pointed out that in Experiment 28, rat 209 escaped infection when exposed to infection by 138 fleas during a period of one day, at the beginning of which the age of infection in the fleas could not have been less than a few hours nor more than five days at the end. Rat 215 became infected by the fleas a day later, when the age of the infection in the fleas could not have been less than two or more than six days.

Putting together the results of this and the last section, it is seen that fleas in which there is a possibility, from the data of the experiment, of the infection being more than five days old, may fail to produce infection, although the subsequent history of those fleas shows them to have been infected effectively, but no infections have been obtained in any experiment of which the data are incompatible with the infection being at least six days old in the fleas that produced the infection.

- (vi) The Infection of the Rat is brought about by the Small Trypanosome-form which is the Final Form of the Development.

This point is scarcely capable of direct proof, since it is impossible to be absolutely certain that when an infection has been produced no other forms of the developmental cycle in the flea were introduced into the rat except the trypanosome-forms. It can, however, be demonstrated in experiments planned for that purpose, that the trypanosome-forms

are present when an infection is produced. Nöller (1912) and Wenyon (1913), have shown that the trypanosome-forms were present in all cases in the fæces with which they infected rats per os.

The following experimental results indicate that the trypanosome-form is the effective agent in infection. In Experiment 35 B twelve fleas were taken at hazard from the infected breeding-cage and put on clean rat 243 for five days (23 : ii : '11 to 28 : ii : '11; Rat 243 was found to be infected on 3 : iii : '11). Ten of the fleas recovered were then dissected (the other two lost); of each flea the stomach was placed on one slide in a drop of salt-citrate solution, the rectum on another slide in another drop. Each stomach and each rectum were then teased up and examined microscopically in order to see if trypanosomes were present in any form; but since this examination had to be performed very rapidly and cursorily and without putting a coverslip over the drop, trypanosomes may have been often overlooked, when they were not present in abundance. Whether trypanosomes could be seen in the fresh specimen or not, each teased-up stomach was inoculated by means of a syringe into a separate clean rat; the rectum was only inoculated if trypanosomes were seen in it.¹ After the drop containing the teased-up stomach or rectum had been drawn up into the injecting syringe the film of moisture left on the slide was fixed with osmic vapour, stained with Giemsa's stain, and carefully searched for trypanosomes. The following are the results obtained with each flea.

Flea (1).—Nothing seen in the fresh stomach or rectum. Stomach inoculated into Rat 279. No infection produced. Nothing found in the preserved film.

Flea (2).—As last, stomach inoculated in rat 280, no infection, nothing found in the films.

¹ The reason for the differential treatment of the stomach and rectum was because we believed, at the time, that infection was brought about by regurgitation of infective trypanosomes through the proboscis from the stomach, and also because the presence of trypanosomes in the rectum is not so easily overlooked as in the stomach.

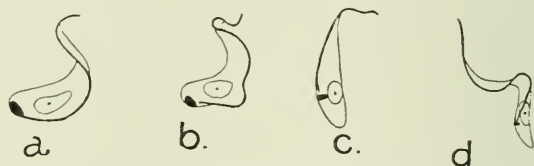
Flea (3).—As last, stomach inoculated into rat 281, no infection, nothing found in the films.

Flea (4).—One sluggish stumpy form, which may have been crithidial or trypaniform, was seen in the fresh teased-up stomach; nothing seen in the fresh rectum. Stomach inoculated into rat 283, result negative. Nothing found in the preserved film of the stomach.

Flea (5).—Nothing seen in the fresh stomach, numerous trypanosomes seen in the rectum. Stomach inoculated into rat 285, result positive. Rectum inoculated into rat 248, result negative. One trypanosome-form and one transitional form found in the preserved film of the stomach (Text-fig. 16, *b* and *c*). Nothing found in the preserved film of the rectum.

Flea (6).—Nothing seen in the fresh stomach; a few forms, some

TEXT-FIG. 16.



Small trypanosome-forms from the stomach-films of fleas 5 and 7 in Experiment 35 B, and flea 5 in Experiment 27 (see text). ($\times 2000$.)

stout and of crithidial appearance and some slender, apparently trypaniform, seen in the rectum. Stomach inoculated into rat 286, result, negative; rectum not inoculated. No films preserved.

Flea (7).—Nothing seen in the fresh stomach or rectum. Stomach inoculated into rat 288, result positive. One trypanosome (Text-fig. 16, *a*) found in the preserved film of the stomach.

Fleas (8), (9), (10).—In each case nothing was seen in the fresh stomach or rectum. The stomachs were inoculated into rats 289, 262, 263 respectively, results in each case negative. Nothing found in the preserved films.

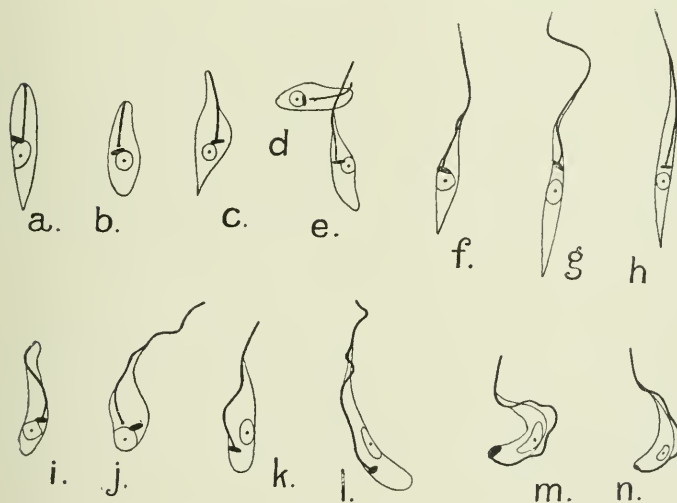
Summary.—In the case of two fleas out of the ten used, the stomachs, when inoculated into clean rats, produced an infection. The final trypanosome-stage was found in both the stomachs that produced infections, but in none of the remaining eight stomachs that produced no infection.

Experiments 27, 29 and 32 were conducted in a different

manner. Fleas taken from the infected breeding-cage were put each on a separate rat and left on it for three or four days. The flea was then recovered (if it could be found), dissected and examined.

Experiment 27.—Flea (5), placed on rat 207 for three days (2 : viii : '10 to 5 viii : '10) produced infection (see Table E). The flea dissected (5 : viii), and one large transitional form found in the slide of the stomach (Fig. 16, *d*).

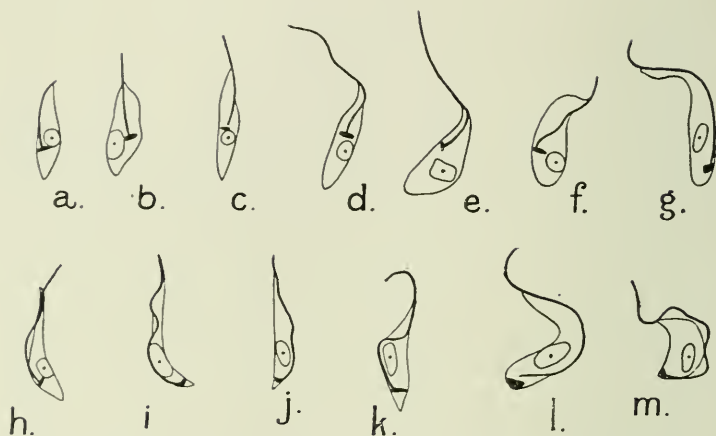
TEXT-FIG. 17.



Various forms (haptomonad, *a-d*, nectomonad, *e-h*, transitional, *i-l*, and trypaniform, *m* and *n*), from the stomach-film of flea 3, Experiment 29 (see text). ($\times 2000$.)

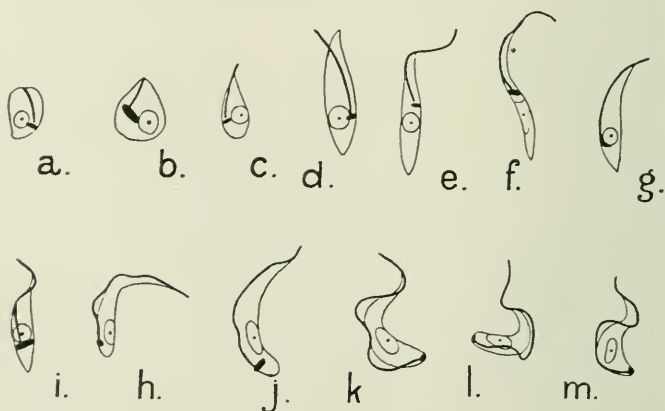
Experiment 29.—Flea (3) placed on rat 212 for three days, (19 : ix : '10 to 22 : ix : '10), recovered and dissected 22 : ix '10 (see Table E). Large clumps of attached forms were seen in the stomach and also free forms; nothing was seen in the intestine, rectum, salivary glands or proboscis. The preparations of the stomach showed crithidial, transitional and trypaniform types in abundance (Text-fig. 17). Rat 212 became infected and first showed trypanosomes in the blood on 28 : ix. Four other fleas in the same experiment failed to infect their rats; in two of these fleas nothing was found, in the third a small

TEXT-FIG. 18.



Various forms (haptomonad, *a, b*, nectomonad, *c, d*, transitional, *e-h*, and trypaniform, *i-m*), from the stomach-film of flea 3, Experiment 32 (g) (see text). ($\times 2000$.)

TEXT-FIG 19.



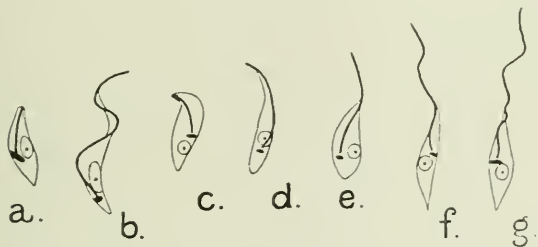
Various forms (haptomonads, *a-d*, nectomonad, *e*, transitional, *f-i*, and trypaniform, *j-m*) from the rectum-film of flea 3, Experiment 32 (h) (see text). ($\times 2000$.)

attached clump was seen in the intestine, nothing in any other part of the flea. The remaining flea was not recovered.

Experiment 32 (g).—Flea (3) placed on rat 241 for four days (3 : xi : '10 to 7 : xi : '10) produced no infection. In the stomach of the flea, dissected 8 : xi, erithidial, transitional and trypaniform types were found (Fig. 18).

Experiment 32 (h).—Flea (3) placed on rat 244 for four days (11 : xi : '10 to 15 : xi : '10) produced an infection (see Table E). The flea was dissected 15 : xi ; nothing was found in the stomach, intestine or salivary glands ; the rectum showed a typical swarming " pile-carpet " infection with all the usual types of form (Text-fig. 19).

TEXT-FIG. 20.



Flea 3, Experiment 32 (i). *a*, haptomonad, and *b*, trypanosome-form from the rectum-film; *c* and *d*, haptomonads, *e-g*, nectomonads, from the film of the intestine (see text). ($\times 2000$.)

Experiment 32 (i).—Flea (3), placed on rat 247 for three days (12 : xi : '10 to 15 : xi : '10), produced no infection. The flea, dissected and examined (16 : xi), showed no trypanosomes in the stomach, but in the intestine were clumps attached behind the pylorus (Text-fig. 20, *c*), and the rectum contained a teeming infection (Text-fig. 20, *a* and *b*) of the usual types.

The cases cited show that when infections were produced the final trypanosome-form was found either in the stomach or rectum ; but it should also be mentioned that we have three instances in which the rat became infected under similar circumstances without our having been able to discover an infection of the flea, which must have been so scanty as to escape detection in our films. On the other hand the experiments also show that the infective form may be present in the

flea without any infection resulting when the flea is on the rat for not more than four days. The failure of the flea to infect in such cases must be correlated with the casual nature of the contaminative method of infection by the fleas, evidently not so sure a method as that of inoculation. It will be shown further (Experiment 39, below) that the period at which the flea becomes infective coincides with the first appearance of the small trypanosomes in the rectum.

- (vii) The Final Infective Form of the Cycle is developed first in the Rectum on the Fifth Day of the Developmental Cycle, but may appear later in the Stomach.

In order to ascertain how soon the trypanosomes, ingested by the flea, attain to maturity in the different parts of the digestive tract of the flea an experiment (Experiment 39) was carried out in the following manner. A number of fleas (about one hundred) were collected from the non-infected breeding-cage, put into test-tubes, with clean sand, slightly damp, and kept there for four days (20:iv:'11 to 24:iv:'11), in order that they should be properly hungry and ready to feed. The fleas were then (24:iv) put into a special flea-proof tin cage with a well-infected rat (No. 259).¹

At regular intervals batches, each of ten fleas, were recovered from rat 259, kept in the test-tubes on sand, and dissected on the following day (to ensure that the fleas had not ingested blood containing trypanosomes for at least a day previous to being dissected). In the dissection of the fleas, the flea was first placed on a slide in a drop of salt-citrate solution and the proboscis removed by cutting through the head in the region of the eyes; the proboscis was then placed in a separate capsule in a small quantity of salt-citrate solution. Very often fæces were extruded when the

¹ Rat 259 was put in with a single flea on 22:iii:'11; trypanosomes were first seen in the blood on 30:iii; multiplication was ended on 4:iv: see Table E, p. 617 above.

head was cut through. When this occurred the carcase of the flea was at once removed to another slide, and the extruded faeces examined microscopically. If trypanosomes were found in sufficient abundance the slide and coverslip were fixed and stained. The carcase of the flea was then opened in the hinder end of the abdomen, and the junction of stomach and intestine cut through behind the Malpighian tubes, after which the portion of the carcase containing the stomach was transferred to another slide. Then the stomach and Malpighian tubules, together with the proventriculus and œsophagus, were removed together and transferred to a second capsule, and the proctodæum (intestine with rectum) to a third. In making these dissections some of the contents of the stomach or rectum escaped on to the slide (the organs being purposely punctured to allow some contents to escape, when necessary). The escaped contents were examined microscopically, and if trypanosomes were found in them in sufficient numbers they were preserved.

After all the 10 fleas of each batch had been dissected in this way the 10 proboscides were inoculated into one clean rat, the 10 stomachs into another, and the 10 recta into a third. In each case the whole of the salt-solution in the capsule was injected also. The stomachs and recta were teased up as fine as possible, and the proboscides crushed up, before injecting them.

The following are the actual injections performed; in the results stated, 0 signifies that the rat inoculated acquired no infection, + that it became infected.

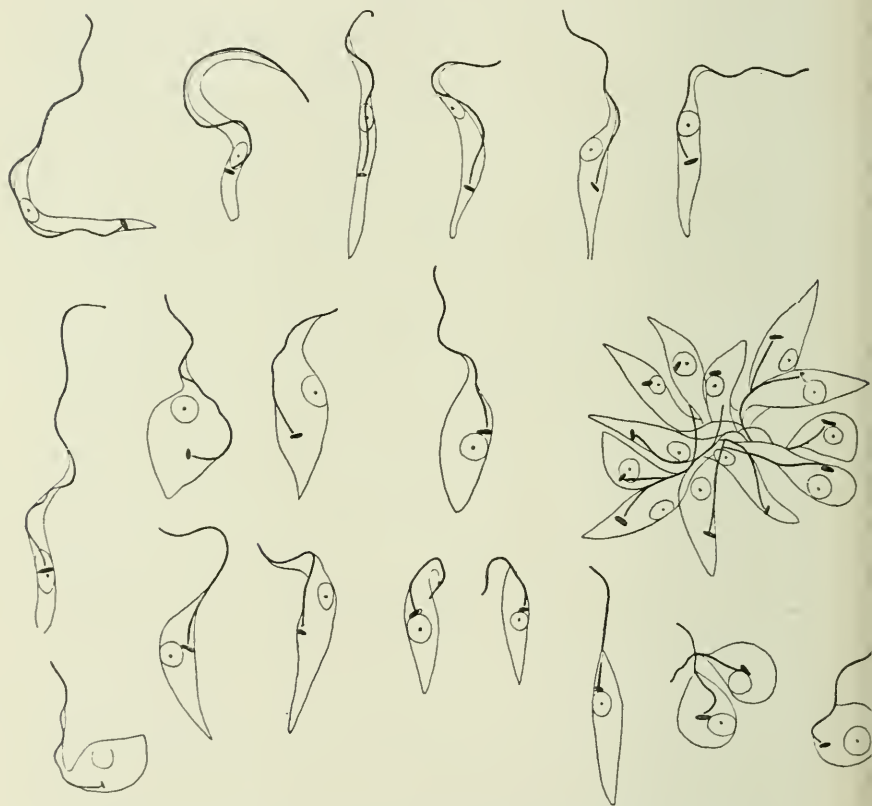
26 : iv : '11.—The 10 fleas recovered on the previous day were dissected; trypanosomes were seen in the stomach, rectum, and extruded faeces of several fleas. All trypanosomes seen appeared to be of quite ordinary type:

10 proboscides injected into rat	289 :	result	0
10 stomachs		299 :	.. 0
10 recta		300 :	.. 0

27 : iv : '11.—The 10 fleas recovered on the previous day were dissected; trypanosomes were seen in the stomach of one, the stomach and extruded faeces of another, in the stomach and rectum of a third, and in

the rectum and extruded faeces, very abundantly, of a fourth; the rectal forms appeared pear-shaped or club-shaped in the living state (Text-fig. 21), but no post-critthidial trypanosome-forms were present:

TEXT-FIG. 21.



Various forms from the rectum and faeces of a flea of the batch of 27:iv:'11. Note that no final trypanosome-forms are present (see text). ($\times 2000$.)

10 proboscides injected into rat 301:	result	0
10 stomachs	" " "	302: " 0
10 recta	" " "	303: " 0

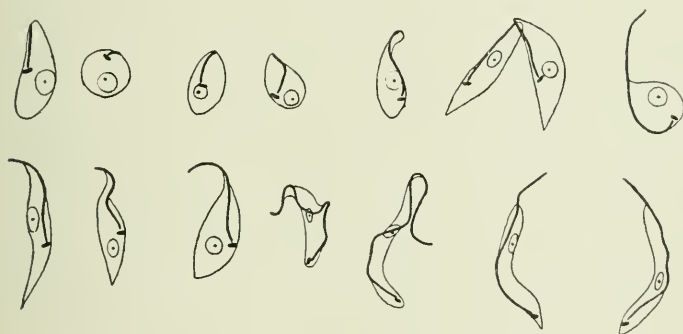
28:iv:'11.—The 10 fleas recovered on the previous day were dissected; trypanosomes were seen in the stomachs of three, and in the stomachs and extruded faeces of two others. The faeces were preserved

in one case where they were seen, but no trypanosomes were found in the preparations:

10 proboscides injected into rat	304:	result	0
10 stomachs	" " "	305:	" 0
10 recta	" " "	306:	" 0

29:iv:'11.—The 10 fleas recovered on the previous day were dissected; trypanosomes were seen in the extruded faeces of one, in the rectum and faeces of another (abundantly) and the rectum of a third (abundantly); of the last two, preparations were made of the rectal contents, and there were found pear-shaped crithidial, transitional, and post-crithidial trypaniform individuals (Text-fig. 22).

TEXT-FIG. 22.



Various forms from the rectum and faeces of two fleas of the batch of 29:iv:'11, Experiment 39 (see text). Note the trypanosome-forms (last 4 figs. to the right, second row). ($\times 2000$.)

10 proboscides injected into rat	307:	result	0
10 stomachs	" " "	308:	" 0
10 recta	" " "	309:	" +

1:v:'11.—The fleas recovered 29:iv were dissected; in one of them, a male, minute crithidial individuals were seen in the stomach contents, but not in the rectal contents; no preparation was made.

10 proboscides injected into rat	310:	result	0
10 stomachs	" " "	311:	" 0
10 recta	" " "	312:	" +

4:v:'11.—The 10 fleas recovered on the previous day were dissected, trypanosomes were seen in the rectum of one, in the extruded faeces of another; no preparation made.

10 proboscides injected into rat	313:	result	0
10 stomachs	" " "	314:	" +
10 recta	" " "	315:	" 0

Summary of Experiment 39.—None of the rats inoculated with organs of the fleas which had been exposed to infection two, three, or four days previously became infected. On the fifth and seventh days inoculation of the recta produced infections, while the inoculations of the stomachs were negative. On the tenth day, on the other hand, inoculation of the stomachs gave a positive, that of the recta a negative result. It is seen, therefore, (1) that the fleas first became infective on the fifth day, when also the post-critlidal trypanosomes were first found in preparations of the rectum; (2) that the developmental forms which produce infection were present in the rectum, but not in the stomach, on the fifth and seventh days; and in the stomach, but not in the rectum, on the tenth day.

(viii) The Developmental Forms of the Trypanosomes in the Flea are not infective when inoculated into the Rat during a period extending from a short time (half an hour?) after being taken up by the Flea until the Developmental Cycle is complete.

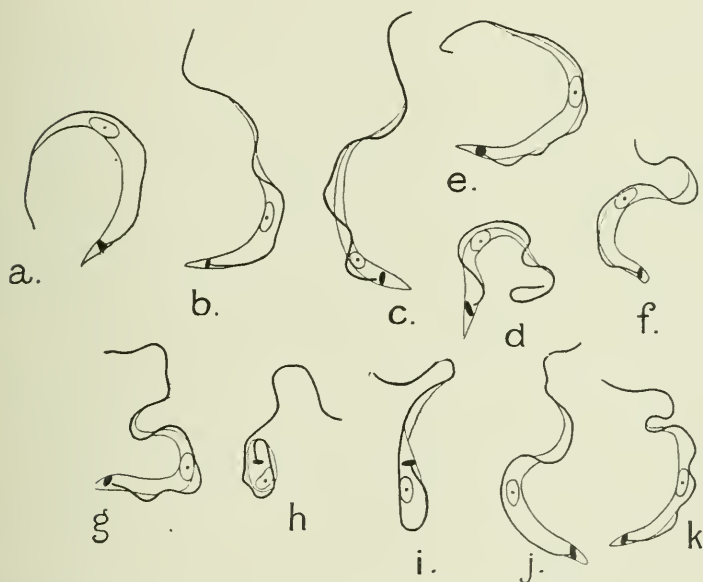
After we had shown, in Experiment 39 (see above), that the trypanosomes in the flea are not infective to the rat after they have been in the flea for two days, and that they do not acquire infectivity for five days after being ingested, we instituted a number of experiments with a view of discovering how soon the ingested trypanosomes lose their power of infecting.

In our first experiment a number of fleas collected from our non-infected breeding-cage and kept hungry for three days (7:xi:'11 to 10:xi:'11), were put on a well-infected rat at 6 a.m. (10:xi:'11) and collected two hours later. They were then dissected in batches, the stomachs of each batch being placed together on the same slide, teased up in a drop of salt-solution, drawn up into an injection-syringe and inoculated into a clean rat. After the drop had been drawn up

into the syringe the film of moisture left on the slide was fixed with osmic vapour and stained with Giemsa's stain, in order to get an idea of the modification, if any, which the trypanosomes had undergone.

The following were the batches dissected and injected:

TEXT-FIG. 23.



Trypanosomes from the stomachs of fleas of the batches of 10:xi:'11 (see text).

(1) Four fleas, injected into rat 323 at 11.35 a.m. The preserved film showed trypanosomes of the ordinary blood-type.

(2) Four fleas, injected into rat 324 at 12.35 p.m. The preserved film showed trypanosomes of the ordinary blood-type (Text-fig. 23, *a. b.*).

(3) Four fleas, injected into rat 325 at 1.30 p.m. The preserved film showed trypanosomes for the most part unmodified, with *n* approximated to *N*, others dwarfed slightly as if beginning to degenerate (Text-fig. 23, *c. d.*).

(4) Four fleas, injected at 2.35 p.m. into rat 326. The preserved film showed trypanosomes of the ordinary blood-type (Text-fig. 23, *e. f.*).

(5) Four fleas, injected at 3.30 p.m. into rat 327. The preserved film

showed trypanosomes mostly modified, some rather lengthened out, others recurved (Text-fig. 23, *g, h, i*).

(6) Three fleas, injected at 4.35 p.m. into rat 327. The preserved film showed trypanosomes mostly unmodified, some rather long (Text-fig. 23, *j, k*).

All the results were negative, since none of the rats became infected. It is seen from the times of feeding and injecting the fleas that none of the trypanosomes had been in the fleas more than ten and half hours (6 a.m. to 4.35 p.m.), or less than three and half hours (8 a.m. to 11.30 a.m.).

After obtaining this result we made a number of other experiments, modifying slightly our method of procedure. Fleas taken from the non-infected breeding-cage were fed under observation on a well-infected rat and the time of feeding noted. The flea was recovered, dissected, and its stomach injected into a clean rat after being teased up in a drop of salt-citrate solution. As before, the film of moisture left on the slide was preserved in some cases.

In some cases also some blood from the infected rat was inoculated subcutaneously into a control clean rat. The controls were not always positive, however, since the subcutaneous method of injection is notoriously less efficient than the intra-peritoneal method, when a small quantity of blood is taken direct from the rat.

27: xi: '11.—Fleas fed under observation on an infected rat after having been kept hungry for three days.

Fleas (1) and (2) fed at 11.35, injected into rat 329 at 12.10 (35 minutes). The preserved film showed trypanosomes quite unmodified.

Flea (3) fed at 11.45, injected into rat 329 at 12.20 (35 minutes). The preserved film showed trypanosomes quite unmodified.

Flea (4) fed at 11.30, injected into rat 330 at 12.30 (1 hour). Trypanosomes in preserved film quite unmodified.

Flea (5) fed at 11.50, injected into rat 330 at 12.50 (1 hour). Trypanosomes seen in the fresh stomach, but film not preserved.

Flea (6) fed at 11.50, injected into rat 330 at 12.50 (1 hour). Film badly preserved.

Flea (7) fed at 10.45, injected into rat 331 at 12.45 (2 hours). Trypanosomes in preserved film quite unmodified.

Summary.

Rat 329 was injected with 3 fleas 35 minutes after feeding.

„ 330 „ „ 3 „ 1 hour „ „

„ 331 „ „ 1 flea 2 hours after feeding.

The results in all three cases were negative. No control rat was injected.

8:xii: '11.—Fleas fed under observation on an infected rat (322).

Fleas (1) and (2) fed at 11.40, injected into clean rat 324 at 12.26 (36 minutes). Active trypanosomes seen in the fresh stomachs.

Fleas (3) and (4) fed at 11.53, injected into rat 324 at 12.37 (44 minutes). Trypanosomes seen in the fresh stomachs.

Fleas (5), (6), and (7) fed at 12.4, injected into rat 324 at 12.42 (38 minutes). Trypanosomes seen in the fresh stomachs.

Fleas (8), (9), and (10) fed at 12.10, injected into rat 324 at 12.47 (37 minutes). Trypanosomes seen in the fresh stomachs.

Flea (11) fed at 12.20, injected into rat 324 at 12.55 (35 minutes).

Summary.—Rat 324 inoculated with the stomachs of eleven fleas fed on rat 322 between 35 and 44 minutes previously. Result negative.

Control rat 323 inoculated at the same time with a small drop of citrated blood from rat 322. Result positive (found to be infected on 15 : xii).

24:i: '11.—Eight fleas, kept hungry for three days previously, were fed under observation on an infected rat (No. 392); the stomachs of six inoculated into clean rat 411.

Flea (1) fed 10 a.m., injected 11 a.m. (1 hour).

Flea (2) „ 10.10 a.m., injected 11.10 a.m. (1 hour).

Flea (3) „ 10.15 „ „ 11.14 „ (59 minutes).

Flea (4) „ 10.20 „ „ 11.20 „ (1 hour).

Flea (5) „ 10.25 „ „ 11.25 „ „

Flea (6) „ 10.29 „ „ 11.29 „ „

Fleas (7) and (8), fed 10.33 a.m. and 10.38 a.m. respectively, were dissected and fixed preparations made of them; numerous trypanosomes, quite unmodified in appearance, were found in them.

Summary.—Rat 411 inoculated with the stomachs of six fleas, each of which had fed on infected rat 392 an hour previously. Result negative.

Control rat 412 inoculated with a small drop of citrated blood from rat 392 at 12 noon. Result negative. (N.B.—Rat 412 put into the infected breeding-cage on 15 : ii : '13 contracted an infection in due course and was therefore not naturally immune.)

11 : ii : '13.—Seven fleas fed on infected rat 402 under observation. The stomachs inoculated into clean rat 414.

Flea (1)	fed 10. 9 a.m.,	injected 11. 7 a.m.	
Flea (2)	„ 10.12 „ „	11.12 „	
Flea (3)	„ 10.17 „ „	11.17 „	
Flea (4)	„ 10.25 „ „	11.25 „	
Flea (5)	„ 10.28 „ „	11.28 „	
Flea (6)	„ 10.37 „ „	11.38 „	
Flea (7)	„ 10.58 „ „	11.58 „	

(In all cases the time of feeding was reckoned from the moment the flea withdrew its proboscis.)

Some of the blood was allowed to escape from the stomach of flea (7) and permanent preparations made; numerous trypanosomes quite unmodified in appearance were found.

Summary.—Rat 414 acquired no infection; control rat 415, inoculated with one drop of blood from rat 402, became infected (trypanosomes first seen, 21 : ii : '13).

25 : ii : '13.—One flea was fed under observation at 10.45 a.m. on infected rat 415; the stomach was inoculated into clean rat 419 at 11.45. Results negative. (The other fleas refused to feed.)

27 : ii : '13.—Eight fleas fed under observation on infected rat 415; the stomachs inoculated into clean rat 419, in each case 59 minutes or an hour after feeding. Result negative.

Control rat 414 inoculated with a drop of blood from rat 415. Result negative (rat 414 was later infected by being put into the infected breeding-cage.)

It will be seen from the foregoing accounts that all experiments, in which infected blood ingested by a flea was inoculated into a clean rat from about half an hour onwards after ingestion by the flea, gave uniformly negative results. The controls were sometimes negative, sometimes positive.

(ix) The Flea, when once it has become infective, remains so for a considerable Length of Time.

This point was dealt with in our preliminary account (1910), when it was shown in two experiments ("C" and "D") that cages of fleas when once rendered infective continued to produce infections for some time without being re-infected. Experiment 22 ("C") was continued for some time after the publication of our paper, but on 28 : i : '10 an infected rat was unfortunately introduced into the cage, an oversight which vitiated all subsequent results, and the experiment was discontinued. During the period prior to this accident, however, the experiment was not open to objection, and produced a result which may be summarised as follows. A cage colonised with 160 clean fleas, into which an infected rat was introduced for three days (24 to 27 : xi : '09), and which produced the first infection between 30 : xi : '09 and 3 : xii : '09, continued to produce infections without being re-infected up to 24 : i : '10, a period of approximately 55 days. It is, therefore, a safe conclusion to affirm that the infectivity persisted in some fleas, or at least one flea, for that period of time.

Later a more exact experiment (Experiment 37) was carried out, in which single fleas were used. To begin with, 10 fleas were taken from the infected breeding-cage and put each on a clean rat for four days; at the end of that time the 10 fleas were recovered and each flea put by itself in a separate test-tube in damp sand for six days. Meanwhile the 10 rats were examined daily, and in due course two of them developed infection, the remaining eight being negative. The eight fleas that gave negative results were returned to the infected breeding-cage.

The two fleas that had been proved experimentally to be infective—henceforth known as Flea "A" and Flea "B"—were then placed singly on a succession of clean rats. That is to say, each flea was placed by itself on a clean rat for so many days, then was recovered and placed on another clean rat for so many days, and so on. The experiments with each

flea were continued until the flea disappeared: that is to say, until the flea could not be recovered when sought for.

The details and results of Experiment 37 have been summarised in tabular form in our preliminary communication (1911), but since we have frequently had occasion to refer to the experiment in the present memoir, we think it worth while to reproduce the table already published.

TABLE G.—Summary of Experiment 37.

Flea "A."

No. of rat.	Flea on the rat.	Result.	Trypanosomes.	
			First seen in blood.	Multiplication ended.
265	14-18:ii:'11	+	24:ii:'11	27:ii:'11
249	24:ii-1:iii:'11	0		
260	1-6:iii:'11	+	15:iii:'11	(Rat died, 16:iii:'11)
291	6-13:iii:'11	0		
293	13-17:iii:'11	0		
295	17-22:iii:'11	+	27:iii:'11	31:iii:'11
249	23-27:iii:'11	0		
291	27:iii-1:iv:'11	+	5:iv:'11	9:iv:'11
293	1-5:iv:'11	0		
249	5-10:iv:'11	0		
262	10-15:iv:'11	0		
296	15-20:iv:'11	+	22:iv:'11	25:iv:'11
263	20-25:iv:'11	+	1:v:'11	5:v:'11
292	25-29:iv:'11	+	1:v:'11	6:v:'11
293	29:iv-4:v:'11	0		
294	4-9:v:'11	?	(Rat died, 9:iv:'11)	
249	9-15:v:'11	0		
(15:v:'11 flea not recovered.)				
Flea "B."				
272	14-18:ii:'11	+	24:ii:'11	28:ii:'11
259	24:ii-1:iii:'11	0		
253	1-6:iii:'11	0		
292	6-13:iii:'11	0		
294	13-17:iii:'11	0		
296	17-22:iii:'11	0		
259	23-27:iii:'11	+	30:iii:'11	4:iv:'11
253	27:iii-1:iv:'11	+	5:iv:'11	7:iv:'11
292	1-5:iv:'11	0		
294	5-10:iv:'11	0		
(10:iv:'11 flea not recovered.)				

From the tabular summary it is seen that flea "A" remained infective from about 15:ii:'11 to about 26:iv:'11—that is to say, for a period of 70 days—and flea "B" from about 16:ii:'11 to about 27:iii:'11—a period of 40 days. We have no data for determining the maximum period of time during which a flea can remain infective. It is, perhaps, not improbable that a flea, once rendered infective, may remain so as long as it lives, but we have no knowledge with regard to the average longevity of the flea. Flea "A" (♀) lived under our care from 14:ii:'11 to 9:v:'11—a period of 84 days; but we have no clue as to its age when it was first taken from the breeding-cage.

- (x) The Trypanosome does not penetrate into the Salivary Glands of the Flea, but is confined, during its whole Development, to the Digestive Tract.

To prove this point we began by dissecting out salivary glands of fleas taken from the infected breeding-cage and examining the glands both in the fresh condition, by teasing them up or crushing them with a coverslip, in a drop of salt-citrate solution, and also by means of fixed permanent smears of the glands. In a large number of glands examined very carefully in this way many yeast-like organisms and other similar bodies were found, but never anything that appeared in the least like any possible stage of a trypanosome. Examination of fluid from the body-cavity gave negative results also.

Thinking that we might have failed in these examinations to obtain an infective flea, we took fleas from the infected breeding-cage, put them singly on clean rats for three days or so, and then recovered and dissected them. The organs of the fleas were examined carefully in the fresh condition, and in some cases permanent preparations were also made of them and laid aside. If a rat became infected subsequently, and so proved the flea put on it to have been infective, the preparations of that flea were searched very carefully.

In this way we were able to examine the salivary glands and other organs of fleas which had been proved experimentally to be infective. The following are the details of the experiments and observations; the sign + signifies that trypanosomes were found in the organs mentioned, while 0 means that none were found:

(1) Flea (♀) put on rat 212 for three days (19 : ix : '10 to 22 : ix '10, Experiment 29). Rat 212 found to be infected 28 : ix ; multiplication ended 30 : ix. Flea dissected 22 : ix ; proboscis 0, salivary glands 0, stomach + (Fig. 17), rectum 0, intestines 0.

(2) Flea (♂) put on rat 223 for four days (10 : x : '10 to 14 : x : '10, Experiment 32a). Rat 223 found to be infected 17 : x ; multiplication

TEXT-FIG 24.



Yeast-like bodies of various kinds from the salivary glands of a flea. They are shown in groups, as they were found in the preparation. ($\times 2000$.)

ended 22 : x. Flea dissected and examined 14 : x ; no trypanosomes were seen in the proboscis, body-cavity, stomach, intestine, rectum or salivary glands : but unfortunately no permanent preparations were made.

(3) Flea (♀) put on rat 233 for five days (20 : x : '10 to 25 : x : '10, Experiment 32e). Rat 233 found to be infected 28 : x ; multiplication ended 31 : x ; scanty infection, with few trypanosomes. Flea dissected and examined 25 : x ; no trypanosomes seen in proboscis, body-cavity, stomach, intestine, rectum or salivary glands ; permanent preparations made of salivary glands, 0. (N.B.—Many yeast-like bodies in the salivary glands, see Text-fig. 24.)

(4) Flea (♂) put on rat 238 for three days (1 : xi : '10 to 4 : xi : '10, Experiment 32f). Rat 238 found to be infected 14 : xi ; multiplication ended 16 : xi. Flea dissected and examined 4 : xi ; no trypanosomes were seen in the proboscis, stomach, intestine, rectum or salivary glands ; permanent preparations made of the stomach and salivary glands, both 0.

From the foregoing experiments it is seen that nothing which could be recognised as a trypanosome was found in the salivary glands of four fleas known to have been infective. In one of the four fleas the salivary glands were examined only in the fresh condition, but in the other three fleas the salivary glands were examined both in the fresh condition and in the permanent preparations.

It will be remarked, however, that no trypanosomes were found in any part of the digestive tract, when examined fresh, in three out of the four fleas, although there can scarcely be any doubt that trypanosomes must have been present. As a matter of fact, a scanty infection of the crithidial or final trypanosome-forms, small in size and sluggish in movement, is easily overlooked in the fresh preparations of the teased-up digestive tract, especially in an organ relatively so large as the stomach, which may be gorged with blood-débris greatly hindering and obscuring the examination. It has been our experience not infrequently that the smaller forms of the cycle have been found scantily in permanent preparations of stomachs in which nothing was seen in the fresh examination. This scarcely applies, however, to organs so minute as the salivary glands, the contents of which can be scanned comprehensively in one field of the microscope. We have, therefore, cited the second flea in spite of the fact that no permanent preparations of the salivary glands were examined.

After having made the negative observations recorded above, the idea occurred to us that any form of the trypanosome found in the salivary glands would probably be a final stage of the cycle, destined to be inoculated by the flea through the proboscis into the rat; and that consequently the examination of fleas which had produced an infection recently would be inconclusive, since in such fleas the salivary glands might be purged of their infection, temporarily at least.

We therefore carried out some experiments in which the object was to determine which organs of the flea contained the infective stages of the trypanosome, by dissecting fleas taken from the infected breeding-cage and injecting their

organs separately into clean rats. Thus a batch of fleas, at least five in number, was taken from the infected breeding-cage, and all the fleas in the batch were dissected at the same sitting. The stomachs of all the fleas were put together in one watch-glass and the salivary glands in another. Each flea has four salivary glands (two on each side of the body), but we did not succeed in every case in dissecting out all four of these minute organs; sometimes only two or three were obtained, or even one only of the four (on foggy mornings); but in every case at least one of the four glands was obtained. In the case of the recta (cut off behind the pylorus and therefore including the greater part of the intestine as well), each was examined microscopically and only kept for injection if seen to contain trypanosomes.

In our earlier experiment (Experiment 33) only the salivary glands were used for injection, with the following results:

TABLE H.—Experiment 33.

Date.	No. of fleas dissected.	No. of salivary glands injected.	Rat.	Result.
17 : xi : '13	11	34	248	0
5 : xii : '10	5	18	248	0

Hence fifty-two salivary glands, dissected out from sixteen fleas taken from the infected breeding-cage, failed to infect a clean rat when injected into it.

Our later experiment (Experiment 34) was carried out more elaborately. In the first place each batch of fleas, after having been collected from the infected breeding-cage, was first put on a clean rat, as a control. Then the fleas were recovered from the control rat and dissected; the salivary glands and stomachs were then injected into two clean rats respectively, and the recta, if anything was seen in them, into a third. The details of Experiment 34 are shown in the following table:

TABLE I.—Experiment 34.

No. of fleas.	Fleas put on control rat.	Fleas recovered from control rat.	Fleas dissected.	No. of salivary glands injected.	No. of rat (s. g.).	Result	No. of stomachs injected.	No. of rat (stom.).	Result.	No. of recta injected.	No. of rat (recta).	Result.	No. of control rat.	Result (control).
6	22: xi: 10 25: xi: 10			24	256	0	6	257	0	1	255	0	239	0
6	12: xii: 10 15: xii: 10			22	256	0	6	257	0	—	—	—	239	0
5	5: i: 11 9: i: 11			19	243*	0	5	245	0	—	—	—	231*	0
6	6: i: 11 10: i: 11			24	246	0	6	247	0	2	259	0	241	0
6	19: i: 11 25: i: 11			24	253*	0	6	259*	0	1	258	0	250	0
6	19: i: 11 25: i: 11			22	260	0	6	261	0	—	—	—	251	0
5	19: i: 11 25: i: 11			15	262	0	5	263*	0	—	—	—	252	0

The rats marked * became infected in subsequent experiments, and were therefore not naturally immune.

From the details summarised in the above table it is seen that no infection was produced by 150 salivary glands taken from forty fleas, divided into seven batches. In three of these batches, comprising eighteen fleas, from which sixty-eight salivary glands were obtained, both the control rats and the rats infected with the stomachs¹ became infected. In a fourth batch, comprising five fleas from which nineteen salivary glands were obtained, the control rat was negative, the rat injected with the stomachs was positive. In a fifth batch, comprising five fleas from which fifteen salivary glands were obtained, the control rat became infected but the stomachs produced no infection; the infectivity of this batch must have been in the recta, which unfortunately were not injected. Thus, omitting the two batches that gave negative results throughout and reckoning only with five batches proved to contain infective fleas, it is seen that 102 salivary glands obtained from twenty-eight fleas produced no infection. These results convinced us finally that the salivary glands of the flea play no part whatever in the transmission or development of the trypanosome, and from this time we paid no further attention to them.

- (xi) The Rat can become infected by eating infected Fleas, but not until the Developmental Cycle of the Trypanosome in the Flea is completed.

The fact that rats can become infected by eating infected fleas must now be considered as well established. It was first stated in print by Strickland (1911), but was then already known to us both from experiments performed by ourselves and from others carried out by Dr. Nicoll at the Lister Institute (see our preliminary report, 1911).

We carried out some further experiments to determine whether the rat could become infected in this way before the

¹It should be noted that the stomachs here included the post-pyloric upper end of the intestine, which, as shown above, is often the site where the trypanosomes establish themselves.

developmental cycle of the trypanosome was completed in the flea. The experiments (Experiment 46) were carried on for a long period, each experiment occupying a week. The following sample is typical of the whole series, all being performed in the same manner and each corresponding stage of the experiment being carried out on the same day of the week.

Sunday, 20:iv:'13.—A large batch of fleas (batch 6) collected from the non-infected breeding-cage the Friday previously were put on a well-infected rat.

Tuesday, 22:iv:'13.—About fifty fleas were collected from the batch (6) and fed to the "two-day rat," clean rat 445. The method was to place the fleas, when collected, on the surface of water in a suitable vessel; then each flea was carefully picked off with a fine forceps, and either it was decapitated on a slide in a drop of water with a needle, or its head was crushed with the forceps. Several fleas so treated were stuck into a pellet of damp bread and given to the rat, previously kept hungry for a time. As a rule the rats when fed in this way ate both the bread and the fleas readily and even greedily.

Thursday, 26:iv:'13.—About fifty more fleas of the infected batch (6) were collected and fed to the "four-day rat," clean rat No. 448, in the same manner.

Saturday, 24:iv:'13.—About fifty more fleas of the infected batch (6) were collected and fed to the "six-day rat," clean rat 432, in the same manner.

On the Sunday following a fresh batch (batch 7) was exposed to infection in the same way. On the Tuesday following the "two-day rat" (No. 445), being found not to have become infected, was fed again with about fifty fleas of batch 7. On the Thursday following the four-day rat (No. 448), not having become infected, was fed again with about fifty fleas of batch 7. On the Saturday following (3:v:'13) the six-day rat (No. 432), which then showed no trypanosomes in its blood, was fed with thirty-four fleas of batch 7. Rat 432 was found, however, to be showing trypanosomes in its blood when examined four days later (7:v:'13); it must have been infected by the fleas of batch 6. For batch 8, in the following week, a fresh clean rat, No. 452, was appointed to be the new six-day rat; it later became infected by the six-day fleas of batch 12.

These experiments were continued in regular routine in the manner described, from 16:ii:'13 to 22:iii:'13, and from 20:iv:'13 to 12:vi:'13, in all thirteen weeks and

thirteen batches. The results may be summarized briefly: No rat fed either with fleas exposed to infection two days previously, or with fleas exposed to infection four days previously, became infected; on the other hand, two of the six-day rats fed with fleas exposed to infection six days previously became infected. It may be inferred, therefore, that rats cannot be infected by eating infected fleas until the infection in them is ripe, that is to say until the developmental cycle of the trypanosome in the flea is complete.

(It may be mentioned here that when any fleas remained over from any of the batches used in this experiment they were fed under observation on clean rats on the Monday, Tuesday or Wednesday following, that is to say, eight, nine or ten days after they had been first exposed to infection (see p. 654, below).)

- (xii) Infection of the Rat is effected contaminatively, by way of the Rat's Mouth, by the Rat licking from off its Fur or Skin the Moist Faeces of Infective Fleas containing the Final Propagative Form of the Cycle.

This mechanism of infection was first demonstrated by Nöller (1912) and fully confirmed by Wenyon (1913) by means of experiments which put the matter beyond all reasonable doubt. Without repeating the experiments of these authors, we tested their results by another method, namely, by exposing rats, muzzled and pinioned, to infection by a large number of infected fleas.

For the purpose of our experiments, we made use in most cases of our infected breeding-cage, in which the fleas were swarming in great numbers, and in which an infected rat is kept habitually, so that the fleas ingest blood containing trypanosomes every time they feed. As a preparation for the experiment the infected rat was removed from the breeding-cage and kept apart, all fleas found on it being carefully cleaned off and returned to the breeding-cage. The breed-

ing-cage was then left without a rat in it for a certain time, sometimes merely from morning to evening, in other cases a day or two, in order to induce hunger in the fleas. Then a clean rat was introduced into the cage for a single night or from morning to evening, after having been muzzled in the following manner: The muzzle was a conical cap of fine wire gauze, with meshes too fine for a flea to pass through. The cap was large enough to cover the whole head, including the ears; the opening of the cone had a broad rim or sleeve of soft cloth, and a draw-tape was run through the free edge of the sleeve, so that by pulling the two ends of the tape the sleeve could be drawn up as tight as required. When fixing the muzzle, it was slipped over the head and then the sleeve was tightened round the neck behind the ears; the two free ends of the tape were then passed downwards and forwards over the chest and backwards under each axilla; each end of the tape was given a single turn round the upper joint of the fore-leg of its side and then passed backwards and upwards to be tied to the other end of the tape from the other side of the body over the back of the rat. In this way, not only was the head of the rat muzzled so that it could not lick itself or eat fleas, but owing to the fore-legs being secured firmly it could not use them to tear off its muzzle, which the rat always makes violent efforts to do, and which, in spite of all precautions, it sometimes succeeds in doing.

The clean rat, having been muzzled in the manner described, and exposed to the attentions of the fleas for a certain time, was removed from the breeding-cage, and before being unmuzzled it was subjected to a cleansing process which consisted of removing all fleas from it and of washing its fur all over thoroughly with a disinfectant (lysol, about 2 per cent.). The liquid used for washing usually became coloured reddish-brown from the faeces deposited on the fur by the fleas. After the rat had been cleansed thoroughly, its fur was dried by holding it close to an ordinary electric lamp and then its muzzle was removed and it was allowed to lick itself as much as it wished, being kept apart from all fleas and its blood examined at regular intervals.

As a variation of the above procedure the muzzled rat was not put into the infected breeding-cage in some instances, but into a bell-jar with a certain number of fleas taken from the infected breeding-cage and previously kept hungry.

At first the experiments were controlled by putting unmuzzled rats into the infected breeding-cage, but it was found superfluous to do this, since in many of our actual experiments the muzzled rats succeeded in tearing off their muzzles and thus furnished improvised but very efficient controls.

The following is a brief statement of the experiments (controls marked *):

(1) Rat 447, muzzled and put into the infected breeding-cage from 4.30 p.m., 23:iv:'13. to 10 a.m., 24:iv:'13. Examined from 28:iv to 30:v. not infected; inoculated later from wild rat and acquired infection in due course.

(2) Rat 449, muzzled, put into infected breeding-cage for night of 25-26:iv:'13. Examined up to 30:v. no infection; put into infected breeding-cage, 30:v. became infected in due course.

(3) * Rat 450, muzzled, put into infected breeding-cage evening of 30:iv:'13: found next morning with its muzzle off; it became infected in due course.

After the above-mentioned experiments had been performed in the manner described, namely, by cleaning and disinfecting the rat before unmuzzling it, a change of procedure was adopted. The rat that had been muzzled and put in the infected breeding-cage was not disinfected when taken out, but, after having been freed from fleas, its fur was merely dried thoroughly by holding it near an electric lamp before unmuzzling the rat and allowing it to lick its fur. This was in order to see whether dried fæces would produce an infection when licked off.

(4) Rat 467, muzzled, put into infected breeding-cage, 8 a.m. to 4 p.m., 16:ii:'14 (the infected rat having been removed from the cage two days previously). Examined up to 5:iii. no infection.

(5) Rat 468, muzzled, put into infected breeding-cage 10 a.m. to 2 p.m., 19:ii:'14. Procedure otherwise same as in last, no infection.

(6) *Rat 469, muzzled, put into infected breeding-cage, 7 p.m., 20:ii:'14; found next morning (8 a.m.) with muzzle off; became infected in due course.

(7) Rat 470, muzzled, put into infected breeding-cage 11.30 p.m., 23:ii:'14, treated as 467, etc.; no infection. Muzzled and put into bell-jar with 200 hungry fleas taken from infected breeding-cage 9 a.m. to 2.30 p.m., 3:iii:'14; no infection.

(8) *Rat 472, muzzled, put into bell-jar with 200 hungry fleas from the infected breeding-cage, 9 a.m., 26:ii:'14; found at 2.30 p.m. with its muzzle off; became infected in due course.

From the data quoted, it is seen that when the experiments were carried out successfully, that is to say, when the muzzle kept on, the rat did not become infected, alike whether its fur was cleaned and disinfected, or merely dried, before it was unmuzzled. But in those cases in which the rat succeeded in ridding itself of its muzzle by its own efforts, it became infected in due course.

It is evident that the sole effect of the muzzle is to exclude infection of the rat by way of its mouth. The way is still open for the rat to become infected through the skin, either (1) by the trypanosomes passing in through the puncture made by the proboscis of the flea, a possibility suggested by Nöller; or (2) by the faeces being rubbed into wounds or abrasions on the skin; or (3) by the small trypanosomes in the faeces penetrating by their own efforts through the skin. But since all the results with rats muzzled efficiently were negative, it is evident that no infection per cutem by any of these possible ways occurred in these experiments and it becomes highly probable that it does not take place naturally in any of these ways. On the other hand, since the muzzle excludes only infection per os, it becomes also probable that the rats that succeeded in tearing off their muzzles were infected in this manner.

It may be mentioned finally that an experiment (Experiment 43) was carried out in which the faeces, collected overnight in a moist glass capsule, of fleas from the infected breeding-cage were injected under the skin of a clean rat, but the result was negative.

- (xiii) Can the Flea infect the Rat by inoculating the Trypanosomes into it through the Proboscis?

The first to throw doubt upon the occurrence of this mode of transmission were Strickland and Swellengrebel (1910, '12), who made a number of attempts to infect rats by feeding infected fleas on them through gauze. Every such experiment gave negative results. We repeated these experiments, and always with the same negative results.

We also carried out a large number of experiments in which fleas taken from the infected breeding-cage were fed on clean rats under observation. Our course of procedure, in its latest and most highly elaborate form, was as follows: ¹

A certain number of fleas, collected from the infected breeding-cage, or fed on an infected rat more than seven days previously, or known experimentally to be infective, were kept in a flask containing some damp sand for three or more days, to make them hungry. When the experiment was about to take place the fleas that it was proposed to use were put each into a separate test-tube. A clean rat was prepared for the experiment by shaving a small region of its skin, usually on the belly, sometimes on the inside of the thigh (a favourite spot for fleas to feed). The rat was then held still by an assistant, with its tonsure upwards. The test-tube was inverted on to the tonsure and at first kept pressed upon it; the flea was thus emptied out on to the shaved area of the skin. At first the flea runs round and round inside the circle formed by the rim of the test-tube, but usually comes to rest very soon, and inserts its proboscis into the skin. In some cases, however, the flea refuses to feed, and either continues to run about or remains perfectly still in one place, presenting a deceptive appearance

¹ We did not adopt Nöller's method of tethering the fleas, since it seemed to us better that the flea should be free and unhampered in its movements, and would then be more likely to feed in a natural way. Our colleagues, Dr. Martin and Mr. Bacot, who were doing experiments at the same time on transmission of plague, also found it unnecessary to tether the fleas.

of being engaged in feeding, but when recaptured and examined it is found to have been merely thinking.

In fleas carefully watched it is not difficult to observe with a hand-lens the penetration of the proboscis into the skin, the rush of blood into the stomach of the flea, and the withdrawal of the proboscis when the flea is replete. From a number of fleas that were timed carefully it was found that the male flea took about $1\frac{1}{4}$ minutes to fill its stomach, the female about $2\frac{1}{4}$ minutes. In all the fleas we have fed under observation we have never once observed the flea to defæcate while feeding, though particular attention was directed to this point. Only in one case, when the flea after feeding succeeded in making good its escape into the fur, it was found to have defæcated there.¹

While the flea is feeding an assistant holds over it a paint-brush dipped in a thick syrupy solution of sugar and water. As soon as the flea has filled its stomach it withdraws its proboscis and makes a rush for the fur of the rat, but as soon as it does so the assistant dabs the paint-brush down on it and catches it. From the paint-brush, to which it sticks, the flea is put in water, which cleans off the sugar-syrup; it can then be dissected or put back in the cage, none the worse for its adventure. If it succeeds in getting into the fur of the rat it must be recaptured, and any fæces it may have deposited must be washed off with a disinfectant.

Fleas that refuse to feed can either be put back in their test-tubes and given another chance on the following day, or dissected and examined as controls.

In the majority of cases the fleas fed under observation were either fleas taken at random from the infected breeding-cage or fleas which had been fed on an infected rat at a

¹ Our experience of the feeding habits of *Ceratophyllus fasciatus* does not agree in the least with the account given by Nöller for *Ctenocephalus canis*. The habits of the two species are evidently quite different. The former lives in the burrows of the rat, and only goes on to the rat in order to feed, while the latter lives more or less permanently in the fur of the dog.

definite time, and of which the age of the infection was known exactly. In a few cases we used fleas which had been put singly on clean rats and had produced an infection, and were therefore known to be infective. Thus rat 367a had a "known infective" flea fed on it on 26 : vii : '12 and again on 29 : vii : '12, and another such flea was fed on the same rat on 3 : viii : '12, but the rat did not become infected (Experiment 44a).

We have in our notebooks records of 150 fleas fed on rats under observation in this way. Since the results were uniformly negative it is quite unnecessary to refer to them in further detail, but it may be of some interest to mention some cases in which the fleas were dissected and examined immediately after the experiment. These were fleas left over from the batches used in Experiment 46 (see above, p. 648), and were therefore, all of them, fleas in which the age of the infection was known.

11 : iii : '13.—Six fleas left over from batch 3; infection of the fleas nine days old. Five fleas fed, in three of which no trypanosomes were seen, in a fourth the stomach contained an infection of crithidial forms, and in a fifth both stomach and rectum contained crithidial forms. (In a sixth flea, which had not fed, both stomach and rectum also contained crithidial forms.)

12 : iii : '13.—Four fleas of the same batch as yesterday; infection ten days old. All fed. In two nothing was seen, the other two had crithidial forms in the rectum.

13 : iii : '13.—Two fleas of the same batch as last; infection eleven days old. One, which fed, had a few crithidial forms in the rectum. (In the other, not fed, nothing was found.)

17 : iii : '13.—Four fleas of batch 4; infection eight days old. One which fed, had a swarming infection of the rectum and a few crithidial forms in the stomach. (Of the three which did not feed all had crithidial forms in the rectum, one in the stomach also.)

18 : iii : '13.—Three fleas of the same batch as last; infection nine days old. One, which fed, had crithidial forms in the stomach. (Of the two which did not feed, in one nothing was found, the other had scanty crithidial forms both in stomach and rectum.)

19 : iii : '13.—Six fleas of the same batch as last; infection ten days old. Four fed. In one nothing was found; in two there were crithidial forms in the rectum only; in the fourth there were crithidial forms in

the stomach only. (Two did not feed. In one of them nothing was found, in the other there were crithidial forms in the rectum only.)

5 : v : '13.—Ten fleas of batch 8; infection eight days old. Five fed. Of these three showed crithidias in the rectum only, one in the rectum and stomach and one was quite negative. (Five did not feed. Of these one had crithidial forms in the stomach only, one in the rectum only, one both in the stomach and rectum, and two were quite negative.)

13 : v : '13.—Six fleas of batch 8, infection nine days old. One fed, five did not; the examination of all the six was negative in result.

14 : v : '13.—Eight fleas of the same batch as last; infection ten days old. Five fed, three did not; the examination in all cases was negative in result.

19 : v : '13.—Six fleas of batch 9; infection eight days old. Only one flea fed; all the six negative.

20 : v : '13.—Six fleas of the same as last; infection nine days old. Four fed, two did not; all six negative.

26 : v : '13.—Six fleas of batch 10; infection seven and a half days old. One fed which was negative. (Of the five which did not feed, one was quite negative, three had crithidial forms in the rectum only, and one both in the stomach and rectum.)

27 : v : '13.—Six fleas of the same batch as last; infection eight and a half days old. Of four that fed, two were quite negative, two had crithidial forms in the rectum. (The two that did not feed had crithidial forms, both in stomach and rectum.)

2 : vi : '13.—Six fleas of batch 11; infection eight days old. Three that fed in two cases crithidial forms in the rectum only, the third was quite negative. (In the three that did not feed, two were quite negative, one had crithidial forms in the rectum only.)

3 : vi : '13.—Six fleas of same batch as last; infection nine days old. Two that fed were both negative. (In the four that did not feed, two had crithidial forms in the rectum only, one in the stomach only, one in both rectum and stomach.)

9 : vi : '13.—Seven fleas of batch 12; infection eight days old. Three fed, two of which had crithidial forms in the rectum only; one was quite negative. (All the four that did not feed had crithidial forms in the rectum, and one of them in the stomach also.)

It is seen from the data that in many cases the fleas that fed on the rats contained copious infections in the rectum, the stomach or both; but not in a single case was any infection produced in the rats fed upon by the fleas. At the present time, therefore, the answer to the question posed at the head of this section must be a very decided negative.

- (xiv) Hereditary Transmission of the Trypanosome from Flea to Flea does not, in our Experience, take place.

In order to obtain, if possible, fleas infected hereditarily, a clean, freshly-prepared breeding-cage was colonised with 648 flea-larvæ taken from the infected breeding-cage at various dates between 23 : ix : '10 and 26 : x : '10. Adult fleas were first seen in the cage on the latter date, subsequently to which 100 more larvæ were added (3 : xi : '10).

Thinking, however, that the larvæ in the infected breeding-cage might possibly infect themselves directly from the fæces of adult fleas in the cage, we also colonised another clean breeding-cage with larvæ newly-hatched from eggs laid by fleas taken from the infected breeding-cage. The method was to take a certain number of fleas from the infected breeding-cage and keep them overnight in a glass capsule containing a glass coverslip at the bottom. In the morning a certain number of eggs were usually found, some on the glass of the capsule, some on the coverslip. The fleas having been returned to the infected breeding-cage, the eggs were carefully removed by means of a soft camel's-hair paint-brush, slightly moistened, from the glass of the capsule and each egg was placed on a small piece of black paper, to which it adhered. At first the eggs, attached either to the coverslip or to the black paper, were put in the new breeding-cage and allowed to hatch there; between 1 : x : '10 and 11 : x : '10 there were sixty eggs introduced in this way, but since they did not all hatch, another method was adopted. The eggs laid were kept in a glass capsule till they hatched, and then the newly-hatched larvæ were put into the breeding-cage. In this way 159 larvæ were introduced into the breeding-cage between 18 : x : '10 and 22 : xi : '10. The time taken by the eggs to hatch varied between six to eight days in October and ten to twelve days towards the end of November (laboratory-temperature).

In the two cages colonised in this way clean rats were kept

for a long time, but no infection was produced in either case.

- (xv) The Trypanosomes in the Blood of the Rat can render Fleas infective very soon after they make their First Appearance in the Blood, before their Multiplication-Period is over.

In their paper on the life-history of *Trypanosoma lewisi* in the rat-louse, Breinl and Hindle (1909) state that they were unable to produce an infection of the louse when it was fed on the rat during the multiplication-period; they state that "during the first stages of infection, so long as dividing and segmenting forms were present in the blood, the trypanosomes taken up by the louse only degenerated." Wishing to find if this was true for the flea also, we did two experiments (Experiments 26 and 28) in which a number of fleas were first fed on an infected rat during the multiplication-period of the trypanosomes. The fleas were then recovered and used to colonise a freshly-prepared flea-cage, into which a clean rat was put. In both cases the result was positive, showing that fleas can become infective after having fed on infected rats in which the trypanosomes are undergoing multiplication.

We then planned and carried out a more elaborate experiment (Experiment 40) to determine how soon a rat infected by fleas can infect fleas again. For this purpose rat 317 was placed for one day (15-16 : v : '11) in the infected breeding-cage after the cage had been kept without a rat in it for three days, to make the fleas hungry. Rat 317 first showed trypanosomes in its blood on 21 : v ; the multiplication-period was ended 25 : v or the following day.

Meanwhile, a succession of eight flea-cages, numbered A to H, were colonised with clean fleas, taken from the non-infected breeding-cage, and rat 317 was put in each cage successively for one day, thus :

Cage A.	colonised	with 70 fleas,	12: v;	rat 317	put in	16-17: v
.. B	..	.. 70	.. 13: v	..	..	17-18: v
.. C	..	.. 50	.. 15: v	..	..	18-19: v
.. D	..	.. 50	.. 16: v	..	..	19-20: v
.. E	..	.. 50	.. 17: v	..	..	20-22: v
.. F	..	.. 50	.. 18: v	..	..	22-23: v
.. G	..	.. 50	.. 19: v	..	..	23-24: v
.. H	..	.. 50	.. 20: v	..	..	24-25: v

After rat 317 had been taken out of each of the cages, a clean rat was put into the cage in its place and left in, with the following result :

Rat 218	put in	Cage A.	17: v;	result	0
.. 264	..	.. B	18: v	..	0
.. 266	..	.. C	19: v	..	0
.. 267	..	.. D	20: v	..	0
.. 268	..	.. E	22: v	..	0
.. 269	..	.. F	23: v	..	0
.. 270	..	.. G	24: v	.. +	Found to be infected, 14: vi.
.. 271	..	.. H	25: v	..	0

From the above data it is seen that rat 317, exposed to infection 15-16: v, did not render any fleas infective before the batch that fed on it in cage G, 23-24: v, seven to nine days after it had been infected and two days after trypanosomes were first seen in its blood, at a time when the multiplication of the trypanosomes was proceeding actively. The late appearance of the infection in rat 270 (the record of which indicates that infection took place about 6: vi, twelve days after it had been in contact with the fleas and thirteen days after the fleas themselves had been exposed to infection), indicates that only a small percentage of the fleas in cage G became infective.

From these experiments we deduce that infection of the flea is not dependent on the presence of special propagative forms of the trypanosome produced late in the rat's blood.

(3) PROBLEMS OF SPECIAL NATURE.

(xvi) The Trypanosomes succeed in establishing themselves in the Flea and rendering it infective to the Rat in only a Small Proportion of the Fleas (*Ceratophyllus fasciatus*) that ingest them.

It has already been pointed out above, in the description of the developmental cycle of the trypanosome, that in the greater number of the fleas fed on infected rats the trypanosomes degenerate and die out completely, and that they succeed in establishing themselves in but a small percentage of the fleas. We have also tested this question experimentally by the method of taking fleas from the infective breeding-cage and putting these fleas on clean rats. At first we carried out such experiments by taking small batches, each of five or six fleas, from the infected breeding-cage and putting each batch separately on a clean rat. Eleven such experiments were performed with the results summarised in Table J, from which it is seen that three batches, each of five fleas, produced one infection, and that eight batches, each of six fleas, produced four infections. In each case, as in many of the subsequent experiments now to be recorded, the fleas were left on the rat three or four days on the supposition, which is probably correct, that a flea will become sufficiently hungry to feed in the course of three days, and on the further supposition, which has now proved to be incorrect, that the infection passes into the rat through the proboscis of the flea.

The results of the experiments summarized in Table J are inconclusive, and could only permit of deductions approximately exact if carried out in great number. When a batch gives a positive result there is no clue as to the number of infective fleas contained in it. Further, it has been brought home to us by subsequent experience that an infective flea often fails to produce an infection. Thus, comparing Table J with Table I, it is seen that the batch of 5:i:'11 failed to infect rat 231 (a rat infected subsequently in another ex-

TABLE J.—Experiments to determine the Percentage of Infective Fleas by taking batches of Fleas from the Infected Breeding-cage and putting each batch on a Clean Rat.

Experiment No.	Date.	No. of fleas in batch.	Fleas left on rat.	Rat No.	Result.	Tryps. first seen.	Multipl. ended.
27 bis	14:ii:'10	5	4 days	186	0	—	—
"	"	5	4 "	187	+	21:ii:'10	26:ii:'10
"	"	5	4 "	188	0	—	—
34	22:xi:'10	6	3 "	239	0	—	—
"	12:xii:'10	6	3 "	239	+	19:xii:'10	22:xii:'10
"	5:i:'11	6	4 "	231	0	—	—
"	6:i:'11	6	4 "	241	+	16:i:'11	19:i:'11
"	9:i:'11	6	4 "	242	0	—	—
"	19:i:'11	6	4 "	250	0	—	—
"	"	6	4 "	251	+	26:i:'11	30:i:'11
"	"	6	4 "	252	+	25:i:'11	30:i:'11

Compare also Table I above (p. 645).

periment), but, nevertheless, the stomachs of this batch, injected into rat 245, produced an infection.

In order to obtain results from which more exact conclusions could be drawn, we experimented in a different manner, acting under the advice of Dr. M. Greenwood, Statistician of the Lister Institute. We took batches of fleas from the infected breeding-cage and put them on clean rats, one flea on each rat. A large number of such experiments were carried out with the results summarized in Table K, from which it is seen that 115 fleas placed each on a clean rat produced but eleven infections, a percentage of 9.56 approximately.

It is evident, however, that the bare numerical results of the experiments summarized in Table K cannot be taken as final, for the following reasons:

(1) The number of days given in the table as the time during which the flea was left on the rat is reckoned from the date the flea was put on the rat to the date on which the flea was sought for. But in many cases the flea when sought

TABLE K.—Infections produced by Fleas taken at Random from the Infected Breeding-cage and put singly on Clean Rats.

Fleas left on the rat.	Date put on.	Negative results.	Positive results.	Total negative.	Total positive.	Percentage of positive.
One day	6:xii:'10	1	—	—	—	—
	12:xii:'10	3	—	—	—	—
	18:xii:'10	2	—	—	—	—
Total	—	—	—	6	—	0
Three days	2:viii:'10	4	1	—	—	—
	19:ix:'10	2	1	—	—	—
	10:x:'10	3	—	—	—	—
	1:xi:'10	2	1	—	—	—
	18:xi:'10	3	—	—	—	—
	19:xii:'10	3	—	—	—	—
Total	—	—	—	17	3	15
Four days	19:ix:'10	2	—	—	—	—
	10:x:'10	2	1	—	—	—
	13:x:'10	3	—	—	—	—
	14:x:'10	3	—	—	—	—
	3:xi:'10	3	—	—	—	—
	11:xi:'10	2	1	—	—	—
	12:xi:'10	3	—	—	—	—
	29:xi:'10	1	—	—	—	—
	14:ii:'11	8	2	—	—	—
	28:ii:'11	9	—	—	—	—
Total	—	—	—	36	4	10
Five days	19:x:'10	3	—	—	—	—
	20:x:'10	2	1	—	—	—
Total	—	—	—	5	1	16.6
Six days	27:i:'11	10	—	—	—	—
Total	—	—	—	10	—	0
Ten days	29:vii:'11	7	1	—	—	—
Total	—	—	—	7	1	12.5
Sixteen days	9:i:'12	9	1	—	—	—
	13:i:'13	7	1	—	—	—
Total	—	—	—	16	2	11.1
Eighteen days	3:iv:'13	5	—	—	—	—
	21:iv:'13	2	—	—	—	—
Total	—	—	—	7	—	0
Grand total	—	—	—	104	11	9.56

for was not found, and it must be supposed that the flea died. If it died a natural death before going on to the rat the result would of course be negative whether the flea was infective or not. If eaten by the rat, an infective flea would probably produce a positive result.

(2) The fact, now established, that the flea does not infect by the puncture of the proboscis, but contaminatively, renders the infection a very casual affair, especially when only one flea is on the rat, and the probability of the infection taking place is relatively low. This is clearly shown by Experiment 37, tabulated above (Table G, p. 640), in which one infective flea, put on seventeen rats successively, over a period of about three months, infected seven of them, and another flea, put on ten rats over a period of about two months, infected only three of them. An analysis of these results is given in Table L, from which it is seen that twenty-seven exposures of rats to infection by two fleas known to be infective produced ten infections, equivalent to 37 per cent. of positive results.

TABLE L.—Infections produced by Two Fleas known experimentally to be Infective and placed singly on Clean Rats.

Flea left on the rat.	Negative results.	Positive results.	Total negative.	Total positive.
Four days	4	4		
Five days	10	6		
Six days	1	—		
Seven days	2	—		
			17	10

A side-light on the problem of the percentage of infective fleas might be obtained from the dissection and examination of fleas six days or more after they have been fed on an infected rat. From Table B it is seen that, of 118 such fleas examined, fifty-three were found to contain stages of the trypanosome; of these twenty-eight had a scanty, twenty-five

an abundant, infection. The value of these data, however, is somewhat uncertain, as an aid to the solution of the problem under consideration.

From the foregoing summary of the data, it is evident that the computation of the percentage of fleas that become infective, of those exposed to infection, is a somewhat complicated statistical problem. We have submitted the data to Dr. Greenwood, who has kindly supplied us with the report appended below, from which it is seen that the percentage of infected fleas lies probably between 5.9 per cent. and 45.7 per cent., the mean being 25.8 per cent.

Report of Dr. M. Greenwood.

The problem it is desired to solve is the following:

Within what limits does the true proportion of infective fleas in the population of which those enumerated in Table K are a sample probably lie?

Of 115 fleas left not more than eighteen days on clean rats, eleven produced infections. But all infective fleas do not produce infections, and, according to Table L, in twenty-seven trials with unquestionably infective fleas only ten produced infections in clean rats. Accordingly, it follows that the proportion of fleas in the first experiment which actually produced infection must be divided by the proportion found in the other experiment to arrive at the ratio of potentially infective fleas, which is the quantity sought. This is—

$$\frac{\frac{11}{115}}{\frac{10}{27}} = .2583 \text{ or } 25.8 \text{ per cent.}$$

But the two ratios from which this result is derived are each subject to errors of random sampling, and it is necessary to compute the "probable error" of sampling to which the final proportion is subject. Since the two proportions are entirely independent one of another, the square of the standard deviation of their ratio is $\frac{s_A^2 \cdot B^2 + s_B^2 \cdot A^2}{B^4}$ where A

is the proportion observed among the 115 fleas, and s_A its standard deviation and B and s_B similar quantities in the case of the twenty-seven trials of the other experiments.

Using this formula we reach 9.84 per cent. as the value of the standard deviation or .67449 times this = 6.64 per cent. for the "probable error." Taking the usual margin, three times the "probable error," the conclusion may be drawn that the real proportion of infective fleas is very unlikely to be beyond the limits 5.9 per cent. and 45.7 per cent.

This is the conclusion which might, I think, legitimately be drawn from the two experiments, but two cautions must be had in mind. The first is that the number of trials in the second Experiment, 27, is rather small, and consequently the application of the customary theory of sampling errors must be made with hesitation. The second caution is that we are using twenty-seven trials with two fleas, not twenty-seven separate fleas, consequently, the two experiments are not strictly in *pari materia*. The two fleas used gave very different proportions of successes, and it might happen that were a larger number of definitely infective fleas used, the factor for division would be substantially modified. The above calculation can naturally give us no information on this point since it proceeds in terms of trials, twenty-seven trials with two fleas being assumed to be the same as twenty-seven trials with twenty-seven fleas, and that the differences do not depend upon the idiosyncrasies of the fleas, but upon the fluctuations of chance, the fleas being used simply as dice or counters.

- (xvii) Can the First Phase of the Development of the Trypanosomes, namely, the Intra-Cellular Multiplication in the Stomach of the Flea continue beyond the Second Feed of the Flea (counting as the First Feed that by which it became infected)?

With regard to this point, it should first be made quite clear that our observations on fleas examined during early

periods of the development show conclusively that the trypanosomes may have disappeared from the stomach, and the rectal-phase may be well started, even so early as 18, 24, or 36 hours after the first feed.¹ A very clear case of this is the stomach mentioned above (p. 555), in which the infection was thirty-six hours old, and which was examined after being cut into a series of sections; no trypanosomes of any kind were found in the stomach, but immediately behind the pylorus were two attached clumps of quite normal crithidial type. On the other hand, in fleas not fed again after the infective feed we have found normal forms of the stomach-phase as late as three, four, or even five days after the first feed. From such observations it is evident that the stomach-phase is of very variable duration, for some reason, and that in some cases it is ended² long before the time when the flea would, under natural conditions, feed again, while in other cases it persists at least up to this time.

The question, therefore, is not, "Does the stomach-phase continue beyond the second feed?" (since it is certain that it is very often ended before the second feed), but "Can it do so?" and with regard to the question so posed it must be pointed out that a single clear instance of the stomach-phase persisting beyond the second flea would suffice to give an answer in the affirmative with certainty; but so long as the

¹ We do not refer to those cases in which the trypanosomes had disappeared from the stomach by degeneration, and in which the rectum was either empty or contained only degenerative forms; but only to those cases in which the presence of true crithidial forms in the intestine or rectum showed that the development of the trypanosome was following its normal course.

² Assuming, that is, that the intracellular multiplication is an essential part of, and takes place invariably in, the normal development of *T. lewisi*; we believe this to be the case, but we are unable to assert that it is so. It is at least within the bounds of possibility that the development may take occasionally a short cut, that is to say, that the trypanosomes may pass on to the rectum, and there establish the normal crithidial phase without undergoing intracellular multiplication in the stomach.

TABLE M.—Experiments to Test the Influence of a Second Feed on the Persistence of the Stomach-phase of the Trypanosomes in the Flea.
A.—Fleas not Fed a Second Time.

	Date of first (infective) feed.	Date of dissection of flea.	Age of infection in the flea.	Contents of stomach.	Contents of rectum.
1.	24 : vi : '12	27 : vi : '12	3 days	A few active trypanosomes of developmental type	Nothing.
2.	"	"	"	Many active trypanosomes and intracellular stages	A few forms, apparently degenerative
3.	"	"	"	A few active trypanosomes and many intracellular stages.	Two seen, apparently degenerative forms.
4.	"	"	"	Trypanosomes free and intracellular	Nothing.
5.	"	"	"	Nothing	Small forms, apparently degenerative.
6.	"	"	"	A few sluggish trypanosomes, apparently degenerative	Small forms, apparently developmental.
7.	"	"	"	Trypanosomes free and intracellular	Developmental crithidial forms.
8.	"	"	"	Nothing	Nothing.
9.	"	"	"	A few free trypanosomes	A few small forms and one "tadpole."
10.	"	"	"	Nothing	A few small forms, apparently developmental
11.	"	28 : vi : '12	4 days	"	A few small forms of crithidial appearance.
12.	"	"	"	"	Nothing.
13.	"	"	"	Trypanosomes free and intracellular	A few crithidial (?) forms.
14.	"	"	"	Nothing	Nothing.
15.	"	"	"	"	Crithidias fairly numerous.
16.	"	"	"	"	Crithidias fairly numerous.
17.	"	"	"	A few trypanosomes free and intracellular	

18.	1 : vii : '12	3 : vii : '12	2 days	Nothing	Degenerative forms.
19.	" "	" "	" "	"	Nothing.
20.	" "	" "	" "	"	Degenerative forms.
21.	" "	" "	" "	"	Nothing.
22.	" "	" "	" "	"	Developmental crithidias.
23.	" "	4 : vii : '12	3 days	"	Degenerative (?) forms.
24.	" "	" "	" "	A few free and intracellular trypanosomes	Degenerative (?) forms.
25.	" "	" "	" "	Nothing	Nothing.
26.	" "	" "	" "	"	Degenerative (?) forms.
27.	" "	" "	" "	A few free trypanosomes	Nothing.
28.	" "	" "	" "	A few small forms	Swarms of crithidias.
29.	" "	5 : vii : '12	4 days	Nothing	Nothing.
30.	" "	" "	" "	"	"
31.	" "	" "	" "	One long trypanosome seen	Crithidial (?) forms.
32.	" "	" "	" "	Active free trypanosomes	Two degenerative (?) forms.
33.	" "	" "	" "	Nothing	Nothing.
34.	" "	" "	" "	A few long trypanosomes	Two active slender forms.
35.	" "	11 : vii : '12	2½ days	A few long trypanosomes	Nothing.
36.	8 : vii : '12	" "	" "	Nothing	A few degenerative forms.
37.	8 : vii : '12	" "	" "	Long free trypanosomes and attached clumps	A few attached pointed forms.
38.	" "	" "	" "	Nothing	Crithidial forms.
39.	" "	" "	" "	A few trypanosomes	Developmental (?) crithidias.
40.	" "	" "	" "	Nothing	Crithidial forms.
41.	" "	" "	" "	"	Crithidial (?) forms.
42.	" "	" "	" "	"	Clump of pointed forms.
43.	" "	12 : vii : '12	3½ days	"	Crithidias swarming.
44.	" "	" "	" "	"	Crithidias.
45.	" "	" "	" "	Trypanosomes free and intracellular	Nothing.
46.	" "	" "	" "	Nothing	"
47.	" "	" "	" "	"	One developmental form.
48.	" "	" "	" "	"	Nothing.
49.	" "	" "	" "	Free trypanosomes	"

TABLE M—(continued).
B.—Fleas Fed a Second Time.

	Date of first (infective) feed.	Date of second feed.	Date of dissection of flea.	Age of infection in the flea.	Contents of stomach.	Contents of rectum.
1.	1 : vii : '12	3 : vii : '12	4 : vii : '12	3 days	Nothing.	Nothing.
2.	"	"	"	"	"	"
3.	"	"	"	"	"	"
4.	"	"	"	"	"	"
5.	"	"	"	"	"	Crithidial forms.
6.	"	"	"	"	"	Nothing.
7.	"	"	5 : vii : '12	4 days	"	"
8.	"	"	"	"	"	"
9.	"	"	"	"	"	"
10.	"	"	"	"	"	"
11.	"	"	"	"	"	"
12.	"	"	"	"	"	"
13.	8 : vii : '12 (night)	11 : vii : '12 (9-10 a.m.)	11 : vii : '12	2½ days	"	Degenerative (?) forms.
14.	8 : vii : '12	11 : vii : '12	"	"	One or two small pear-shaped forms (post-pyloric (?))	A few crithidial forms.
15.	"	"	"	"	Nothing.	Nothing.
16.	"	"	"	"	"	Crithidial forms.
17.	"	"	"	"	"	Nothing.
18.	"	"	"	"	"	Degenerative (?) forms.
19.	"	"	12 : vii : '12	3½ days	"	Crithidial forms.
20.	"	"	"	"	"	Nothing.
21.	"	"	"	"	"	A few crithidias.
22.	"	"	"	"	"	A few degenerative (?) forms.
23.	"	"	"	"	"	Crithidias.
24.	"	"	"	"	"	A few pointed forms.
25.	"	"	"	"	"	Pointed forms.

26.	8 : vii : '12	11 : vii : '12	12 : vii : '12	3½ days	Nothing.	A few pointed forms.
27.	"	"	"	"	"	Nothing.
28.	24 : vi : '12	27 : vi : '12	28 : vi : '12	4 days	"	"
29.	"	"	"	"	"	"
30.	"	"	"	"	"	Numerous crithidias.
31.	"	"	"	"	"	Nothing.
32.	"	"	"	"	"	A few crithidial (?) forms.

N. B.—When a query (?) is affixed to the statement concerning the contents of the rectum, it signifies that the determination of the nature of the forms seen in the fresh state was not quite certain and that it was not confirmed by the examination of permanent preparations.

ANALYSIS OF THE RESULTS.

SERIES A.

No trypanosomes seen in 11 fleas.

Trypanosomes seen only in the rectum in 18 fleas; in 4 of the cases doubtfully, in 3 certainly, of degenerative type; in 3 of the cases doubtfully, in 8 certainly, of developmental type.

Trypanosomes seen only in the stomach in 6 fleas.

Trypanosomes seen both in the stomach and rectum in 14 fleas; the rectum contained in 5 of these fleas forms doubtfully of degenerative type; it contained in 4 of them forms doubtfully, in 5 of them forms certainly, of developmental type.

SERIES B.

No trypanosomes seen in 18 fleas.

Trypanosomes seen only in the rectum in 13 fleas; in 6 of these fleas doubtfully of degenerative type; in 1 doubtfully, in 6 certainly, of developmental type.

Trypanosomes seen both in stomach and rectum in 1 flea; in this case all the forms seen were crithidial in character, and those seen in the teased-up stomach may have been post-pyloric in position.

SUMMARY.

A.—In 49 fleas not fed a second time, trypanosomes were found in the stomach in 20 cases, and were not found in 29 cases, in 11 of which they appeared to have disappeared altogether in the flea.

B.—Counting only those cases in which the rectum certainly contained developmental forms—namely, 7 fleas—the typical stomach-forms were absent in all.

answer is in the negative, it cannot be regarded as certain, but only as possessing a greater or less degree of probability.

In order to test this point we fed batches of fleas on infected rats and then divided each such batch usually into two batches, A and B. Batch A in each such case was kept starved until it was examined; batch B was fed again before being examined. In cases where fleas of batch B were found on examination not to have availed themselves of the chance of feeding, they were reckoned in batch A. Sometimes the original batch was not divided, but treated as a whole either as an "A" (not re-fed) or "B" (re-fed) batch.

The results of these experiments are tabulated in Table M, which seems at first sight decidedly in favour of the conclusion that the trypanosomes cannot persist beyond the second feed of the flea. It is seen that in forty-nine fleas not fed again after the infective feed, trypanosomes were present in twenty cases; in thirteen of the cases the trypanosomes were of the long stomach type and in eight cases intracellular forms were seen. On the other hand, in thirty-two fleas examined after having been fed a second time, the typical multiplicative stomach-phase was not present in a single instance. Unfortunately, the force of these figures is rather weakened by the fact that, of the fleas fed a second time it can only be asserted positively in seven cases that the rectum contained true developmental crithidial forms and that the developmental cycle was in these cases a "going concern," so to speak. While the figures make it probable, to a certain degree, that the stomach-phase, if it persists up to the time of the second feed, must come to an end then, this conclusion cannot be considered established and must remain a point for further investigation.

If it be true that the stomach-phase cannot persist beyond the second feed, we may enquire how such a result is brought about. It is intelligible that a fresh meal of blood might sweep on all free, extracellular trypanosomes from the stomach towards the rectum, but this would not account for the disappearance of the intracellular forms. It has been

mentioned above that in some insects the epithelium of the mid-gut is regenerated completely after each meal, and we stated further that we were not in a position either to affirm or to deny that, in the case of the flea, the regeneration of the stomach-epithelium takes place in regular correlation with the feeding. If it were so, however, it would become quite intelligible why a second feed should put an end to the intracellular multiplication in the stomach.

(xviii) Starvation of the Flea during the Incubation Period of the Cycle does not inhibit, nor does it necessarily retard, the Developmental Cycle of the Trypanosome in the Flea.

Experiment 45.—A batch of about 250 clean fleas, having been collected from the non-infected breeding-cage and kept hungry for three days, were put (22: vii: '13) on a well-infected rat, for about twenty-four hours. The next day 150 of the fleas were recovered and kept in a flask containing some damp sand.

Two days later (25: vii), thirty of these 150 fleas in the flask were put into a freshly-prepared bell-jar A with a clean rat 368. The next day (26: vii) rat 368 was removed from bell-jar A, all the fleas on it being cleaned off carefully and put back into the bell-jar. Rat 368, examined regularly up to 26: viii, did not become infected.

Two days later (28: vii), clean rat 368a was put into bell-jar A, containing the fleas that had been in contact with rat 368. On the same day, thirty more fleas from the flask were put into bell-jar B with clean rat 369; another thirty in bell-jar C with clean rat 370; and another thirty in bell-jar D with clean rat 371. It will be remembered that the fleas in the flask had been exposed to infection for one day (22-23: vii), and kept without food since then; consequently, bell-jars B, C, and D were colonised each with thirty fleas that had been exposed to infection between five and six days previously and starved since then.

The next day (29: vii), rat 368a was removed from bell-jar A and all fleas recovered from it put back into the bell-jar. Rat 368a did not acquire infection. The same day rats 369, 370, and 371 were removed from bell-jars B, C, and D, all fleas being recovered from them and put back into the respective bell-jars. Rats 369 and 370 did not become infected; rat 371, on the other hand, became infected in due course (see Table C, p. 616).

Two days later (31: vii), rat 368b was put into bell-jar A, rat 369a into bell-jar B, rat 370a into bell-jar C, and rat 371a into bell-jar D.

The next day (1 : viii), rats 369a, 370a and 371a were removed from the bell-jars B, C, and D, and the fleas on them carefully recovered and put back into their respective bell-jars. Rat 368b was left in bell-jar A. In the sequel rats 368b, 369a, and 370a did not become infected; rat 371a showed infection in due course.

Two days later (3 : viii), clean rats 369b, 370b and 371b were placed in bell-jars B, C, and D, and left in till they should become infected. Rat 369b did not become infected; rats 370b and 371b became infected in due course.

The results of the experiment may be summarised in the following manner. We start with four batches (A, B, C, D), each of thirty fleas, which had been exposed to infection on the same rat for one day (22 : vii to 23 : vii).

(1) Batch A (bell-jar A) :

Put on rat 368 from 25 : vii to 26 : vii (about three days after exposure to infection); result negative.

Put on rat 368a from 28 : vii to 29 : vii (about six days after exposure to infection); result negative.

Put on rat 368b, 31 : vii (about nine days after exposure to infection), and left on the rat; result negative.

This batch therefore did not become infective at all.

(2) Batch B (bell-jar B) :

Starved for five days (23 : vii to 28 : vii), then put on rat 369 from 28 : vii to 29 : vii (about six days after exposure to infection); result negative.

Put on rat 369a from 31 : vii to 1 : viii (about nine days after exposure to infection); result negative.

Left in with rat 369b on 3 : viii; result negative.

This batch therefore did not become infective at all.

(3) Batch C (bell-jar C) :

Starved for five days, then put on rat 370 from 28 : vii to 29 : vii (about six days after exposure to infection); result negative.

Put on rat 370a from 31 : vii to 1 : viii (about nine days after exposure to infection); result negative.

Left in with rat 370b on 3 : viii; result positive (the examinations of the rat indicate that infection took place between 4 : viii and 7 : viii).

This batch therefore became infective.

(4) Batch D (bell-jar D) :

Starved for five days, then put on rat 371 from 28 : vii to 29 : vii (about six days after exposure to infection) ; result positive.

Put on rat 371a from 31 : vii to 1 : viii (about nine days after exposure to infection) ; result positive.

Left in with rat 371a on 3 : viii ; result positive.

This batch evidently became strongly infective.

Remarks.—From the above summary it is seen that batch A (not starved) and batch B (starved) failed to become infective, while batches C and D (both starved) became infective.

Batch C did not produce its first infection before 3 : viii ; that is to say not until eleven or twelve days, at least, after exposure of the fleas to infection. It is not legitimate, however, to conclude from this that the developmental cycle of the trypanosome was retarded, since it has been shown above that infective fleas often fail to infect. The fleas may very well have been infective when placed in contact with rats 370 and 370a, but they were in contact with these rats for only about twenty-four hours. The most probable explanation for the two failures to infect is that only a small number of fleas in this batch were infective.

Batch D produced its first infection between 28 : vii and 29 : vii, and since it was exposed to infection between 22 : vii and 23 : vii it follows from these figures that the incubation-period—that is to say, the length of time taken by the developmental cycle of the trypanosomes—must have been between five and seven days. We are justified therefore in concluding that the trypanosomes in this batch went through a cycle of perfectly normal duration. The further fact that this batch never failed to produce infection during the time the experiment was carried on, indicates that the trypanosomes went through their cycle and established themselves successfully in a relatively large number of the fleas.

To conclude : Batches C and D show that starvation of the

fleas during the incubation-period does not inhibit the development of the trypanosomes; and batch D shows further that the development is not necessarily retarded by starvation.

- (xix) Starvation of the Flea following immediately on an Infective Feed favours the Establishment of the Haptomonad Phase in the Rectum, while Starvation begun after the Incubation-Period in the Flea is over favours Migration to the Post-Pyloric End of the Intestine and the Establishment of the Haptomonad Phase there.

Experiments 49 and 50 were carried out with the object of ascertaining what effect, if any, varying food-conditions might have on the incidence and location of the established haptomonad phase in the flea's gut.

Experiment 49.—21 : iii : '14.—A number of fleas collected from the non-infected breeding-cage two days previously were put into a bell-jar with a well-infected rat at eight a.m., and were recovered again at twelve noon. They were then divided into two batches. Batch A, consisting of fifteen fleas, was put into a flask with moist sand at the bottom. Batch B (about forty fleas) was put into a bell-jar with a clean rat (rat 477).

26 : iii : '14.—Five fleas of batch A and four of batch B were dissected and examined.

Of batch A four were positive, one was negative. Of the four positive three showed developing forms of the trypanosomes in both the stomach and the rectum. Two of the three showed large numbers in both stomach and rectum, and in one of the stomachs intracellular forms were found. The fourth positive showed a haptomonad infection in the rectum.

Of batch B only one of the four dissected showed trypanosomes, and these were found free in the stomach-slide.

27 : iii : '14.—Eight fleas of batch A and eight of batch B were dissected and examined.

Of batch A seven were positive; one was negative. Of the seven positive one showed long active forms in the stomach and haptomonads in the rectum, while six showed developing forms in the rectum only—three scanty and three in fair numbers attached mostly round the

rectal surface of the projecting intestine, but in other parts as well. Of batch B only one was found infected, and it showed haptomonads attached about the middle region of the rectum.

The remaining fleas of batch B were now divided into two batches—batch A1 and batch B1. Batch A1, consisting of fifteen fleas, was put into a flask with moist sand at the bottom, and batch B1 was left in the bell-jar with rat 477.

4 : iv : '14.—Rat 477 was removed from the bell-jar and clean rat 478 was put in its place with batch B1.

Four fleas of batch A1 and four of batch B1 were dissected and examined.

Of batch A1 three were positive and one was negative. Of the three positive two showed trypanosomes in abundance in the post-pyloric region of the intestine and nowhere else. In these the trypanosomes were long and slender, and some were club-shaped; while in a third, which showed one or two in the rectum also, there was a swarming infection of haptomonads, as well as long, slender and club-shaped forms in the post-pyloric region. The fourth flea was negative.

Of batch B1 all were negative.

The remaining fleas of batch A1 were now allowed to feed on a clean rat for a short time.

9 : iv : '14.—Five fleas of batch A1 and four of batch B1 were dissected and examined.

Of batch A1 only one flea was positive, and it showed a fair number of slender trypanosomes in the post-pyloric region and nowhere else. Of batch B1 all were negative.

11 : iv : '14.—The remaining fleas of batch A1 were allowed to feed on a clean rat for a short time.

16 : iv : '14.—Five fleas of batch A1 and five of batch B1 were dissected and examined.

Of batch A1 two were positive and three were negative. The two positives showed large numbers of trypanosomes, some free, long and active, some club-shaped, and others were small, round and pear-shaped, in clumps and attached so as to form a lining to the wall of the gut. All were in the post-pyloric region and nowhere else. Of batch B1 all five were negative.

Rat 477 became infected in due course. Rat 478 never became infected. This agrees with results of the examinations.

Experiment 50.—20 : v : '14.—A number of fleas collected from the non-infected breeding-cage two days previously were put into a bell-jar with a well-infected rat late in the evening, were left overnight, and were recovered next morning. They were then divided into two batches. Batch A was kept in a flask with moist sand at the bottom.

Batch B was put into a freshly-prepared bell-jar with a clean rat (rat 496).

26 : v : '14.—Five fleas of batch A and five of batch B were dissected and examined.

Of batch A three were positive and two were negative. Of the three positive two showed haptomonads in the rectum, one being a swarming infection, and the third showed many long, active forms in the post-pyloric region only.

Of batch B all were negative.

28 : v : '14.—Seven fleas of batch A and seven of batch B were dissected and examined.

Of batch A five were positive and two were negative. Of the five positive four showed developing forms in the rectum only, and of these two were swarming infections. The fifth showed haptomonad infection of the rectum, and also free active forms in the stomach.

Of batch B only one of the seven was positive, and it showed a scanty infection of the rectum only.

The remaining fleas of batch B were now divided into two batches—batch A1 and batch B1. Batch A1 was put into a flask with moist sand in the bottom. Batch B1 was put into a bell-jar with a clean rat (rat 497).

2 : vi : '14.—Fourteen fleas of batch A1 and fourteen of batch B1 were dissected and examined.

Of batch A1 four were positive and ten were negative. Of the four positives one showed haptomonads on the rectal surface of the projecting intestine and three showed trypanosomes (one swarming) in the post-pyloric region and nowhere else.

Of batch B1 five were positive and nine were negative. Of the five positives one which had its stomach full of red blood showed a scanty infection in the rectum only. The other four showed infection in the post-pyloric regions only. Of these two (females) had small ova and their stomachs were empty. The remaining two had a fair quantity of brownish-coloured blood-débris in their stomachs. Rats 496 and 497 became infected in due course.

Summary.

A.—Fleas starved from immediately after the infective feed, dissected and examined five and six days after the infective feed.

No. of experiment.	Number of fleas examined.	Number infected.	Site of infection.	Remarks.
49	13	11	Stomach and rectum, 4; rectum only, 7	Intracellular forms in one stomach 3 scanty, 4 swarming— infections mostly in upper part of rectum
50	12	8	Stomach and rectum, 1; rectum only, 6; post-pyloric only, 1	3 scanty and 3 swarming

B.—Fleas that were put into bell-jar immediately after the infective feed, along with clean rat, on which they could feed at any time. Dissected and examined five and six days after the infective feed.

No. of experiment.	Number of fleas examined.	Number infected.	Site of infection.	Remarks.
49	12	2	Stomach only, 1 Rectum only, 1	— Haptomonad infection in middle region.
50	12	1	Rectum only, 1	—

A1.—Fleas in which starvation was begun six days after the infective feed. During the six days the fleas had been fed on clean rats. Those of Experiment 49 were dissected and examined fourteen, nineteen, and twenty-six days after the infective feed. Those of Experiment 50 were dissected and examined thirteen days after the infective feed.

No. of experiment.	Number of fleas examined.	Number infected.	Site of infection.	Remarks.
49	14	6	Post-pyloric only, 5; post-pyloric and rectum, 1	Swarming haptomonad infection in 2.
50	14	4	Rectum only, 1; post-pyloric only, 3	Pile carpet infection upper part; swarming haptomonad infection in 1.

B1.—Fleas kept with clean rats in bell-jar ever since the infective feed. Dissected and examined as in A1.

No. of experiment.	Number of fleas examined.	Number infected.	Site of infection.	Remarks.
49	13	0	—	—
50	14	5	Rectum only. 1; post-pyloric only, 4	Stomach full of red blood; ¹ stomach empty and ova small in 2; stomach contained fair quantity of blood-débris in 2.

These results seem to throw light on the important function that the nectomonad forms described as occurring in the established rectal-phase may have in maintaining the infection in the flea. The optimum food-conditions for the establishment of the haptomonad stage seem to lie somewhere between abundance and poverty, and between partial and complete digestion of the blood-supply. More extended

¹ Although the fleas of batches B and B1 in both experiments had the chance of feeding on clean rats at any time from immediately after the infective feed onwards, all may not have equally availed themselves of the opportunity. It is certain, in fact, from the condition of the ova and of the stomachs of two females of batch B1 that showed post-pyloric infection, that they, for some reason not ascertained, had starved in the midst of plenty; and these should really be transferred to batch A1. The remaining two fleas of batch B1 that showed post-pyloric infection had evidently fed, but not quite recently. The other infected flea of batch B1 had its stomach distended with red blood, and in it the infection was in the rectum only.

In order to test the infectivity of batches A1 and B1 in Experiment 50, twelve fleas of each batch were put on clean rats, two fleas to each clean rat. The result was that two of the six rats belonging to batch A1 became infected, while none of the six belonging to batch B1 became infected. This may indicate that a period of starvation heightens the infectivity of infected fleas, perhaps by inducing increased production of the final propagative forms of the cycle; but further experiments would be required to justify such a deduction.

and varied observations are required, but so far as these experiments go they show that the incidence, location, and continued existence of the haptomonad stage in the flea's gut depend to a large extent on the food-supply. When, under conditions of partial starvation, a sufficient supply of nourishment cannot be obtained in the rectum, the haptomonad stage, if established there, would die out, and the flea would lose its infection were it not that the nectomonads produced in the rectum migrate forwards and re-establish this stage nearer to the food-supply. In like manner it may be assumed that when the food-supply in the post-pyloric end of the intestine becomes continuously too rich and abundant, the nectomonads produced there migrate backwards to the rectum and so the balance is maintained and the infection in the flea is kept up.

LISTER INSTITUTE,
June, 1914.

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DESCRIPTION OF PLATES 36 TO 45,

Illustrating Mr. E. A. Minchin and Dr. J. D. Thomson's paper on "The Rat-Trypanosome, *Trypanosoma lewisi*, in its Relation to the Rat-Flea, *Ceratophyllus fasciatus*."

(In all cases where the age of a trypanosome is stated, it is to be understood as reckoned from the first infective feed of the flea, or, in cases where the fleas were left on the infected rat for some days, from the time when the fleas were first given the chance of feeding on it.)

PLATE 36.

[Various stages of the stomach-phase in film-preparations fixed with osmic vapour and stained with Giemsa's stain; drawn with the camera lucida at a magnification of 3000.]

Figs. 1-12.—Free (extracellular) trypanosomes.

Figs. 1 and 2.—Six hours; fig. 1, practically unmodified; fig. 2, slightly modified in structure.

Figs. 3 and 4.—Twelve hours.

Figs. 5 and 6.—Eighteen hours.

Figs. 7-10.—Twenty-four hours.

Figs. 11 and 12.—Forms of crithidial structure from an abnormal flea (see p. 519); fig. 11, a free specimen; fig. 12, a couple adhering together.

Figs. 13-17.—Recurved forms.

Figs. 13, 14.—Twelve hours; both from the same preparation, but 14 is flattened out by having dried up before fixation, while 13 is normal.

Figs. 15-17.—Twenty-four hours.

Figs. 18-20.—Forms in which the multiplication of the nuclei have begun without the trypanosomes having assumed the recurved form (or perhaps forms which have straightened themselves out again secondarily); twenty-four hours, all from the same preparation. In fig. 19 the trypanosome is enclosed in the remains of the host-cell.

Figs. 21-23.—Rolled-up forms with 2 nn and 2 NN each; from the same preparation as the preceding.

Figs. 24-29.—Rolled-up forms with *n* and *N* still single, in 27 beginning to divide. Figs. 24 and 25 are from the same preparation as the preceding; figs. 26-29 are also twenty-four hours; the specimen in fig. 26 is evidently enlarged artificially by having dried before fixation.

Figs. 30-37.—Multiplication of nuclei to 2 or 3 *nn* and 2 *NN*.

Fig. 38.—Small sphere with 4 *nn* and 4 *NN*.

Fig. 39.—Stage with 3 *nn* and 2 *NN*. N.B.—Figs. 35-39 are all from the same preparation, showing the intense staining and consequent opacity of the body characteristic of the intracellular stages; only the principal flagella can be made out clearly, the daughter-flagella being scarcely, or not at all, visible. Twenty-four hours.

Fig. 40.—Sphere with 3 *nn* and 3 *NN*, from the same preparation as fig. 26; evidently enlarged by flattening due to drying. Twenty-four hours.

Fig. 41.—A large sphere containing 13 *nn* and 13 *NN*, and two others; the stain is over-extracted and the flagella are not seen. These specimens are from the same stomach as figs. 88-94 on pl. 37, in all of which flagella are plainly seen. Thirty-six hours.

Fig. 42.—Two very large tailed spheres from the same preparation as figs. 18-25, 30, 33, 34; the flagella cannot be made out. Twenty-four hours.

Figs. 43-46.—Small rolled-up forms, possibly in process of degeneration, from the same preparation as the last.

PLATE 37.

[Various stages of the stomach-phase from films preserved in sublimate mixtures—sublimate-acetic, Schaudinn's fluid, or Maier's fluid—and stained with iron-hæmatoxylin, drawn with the camera lucida at a magnification of 3000.]

Figs. 47-68.—Free (extracellular) trypanosomes, except 58 and 59.

Figs. 47, 48.—Six hours; fig. 47, quite unmodified; fig. 47, slightly modified.

Fig. 49.—Eighteen hours.

Figs. 50-55.—Twenty-four hours.

Figs. 56-59.—Three days, all from the same preparation. Figs. 58 and 59 are evidently early stages of the intracellular multiplication.

Fig. 60.—Four days.

Figs. 61-68.—From an abnormal flea taken from the infected breeding cage (see p. 519). Every possible grade of transition occurs in the preparation from quite ordinary forms, such as fig. 61, to large crithidial

forms, such as fig. 66. and of the latter some occur in couples adhering together, as in figs. 67 and 68.

Figs. 69-73.—Recurved forms, twenty-four hours; in the specimen shown in fig. 72 the flagellum appears to have become torn away from the body.

Fig. 74.—First division of *n* in a rolled-up form.

Fig. 75.—Recurved form with 2 *nn* and 2 *NN*; forty-eight hours.

Figs. 76.—A rolled-up form in which the flagellum has become untwisted from the body; from the same preparation as the last.

Figs. 77, 78.—Two rolled-up forms from the same preparation, twenty-four hours.

Figs. 79-81.—Twenty-four hours, all from the same preparation; fig. 79, *n* divided, daughter-flagellum arising. *N* beginning to divide; figs. 80 and 81, similar stages.

Figs. 82-86.—Twenty-four hours, all from the same preparation; figs. 82, 83, each 2 *nn*, 1 *N*; fig. 85, 2 *nn*, 2 *NN*; fig. 84, 3 *nn*, 2 *NN*; fig. 86, tailed sphere with 8 *nn*, 8 *NN*.

Fig. 87.—Recurved form, twenty-four hours.

Figs. 88-94.—Various stages, all from the same preparation and from the same flea as fig. 41 on Pl. 36. Thirty-six hours.

Fig. 95.—Small sphere with 3 *nn* and 3 *NN*; twenty-four hours.

Fig. 96.—Large, nearly ripe sphere containing about ten trypanosomes, shown also in the photograph on Pl. 44, fig. 318; forty-eight hours.

PLATE 38.

[Epithelial cells of the stomach and stages of the stomach-phase from sections of the flea's stomach, stained with Giemsa's stain (with the exception of figs. 260 a, 261 a, 262 a, 263 a, and 264, for which see description of Pl. 42). Figs. 99-103 were fixed with Flemming's fluid, but all the rest were fixed with Maier's fluid. Drawn with the camera lucida at a magnification of 2000.]

Figs. 97, 98.—Epithelial cells; fig. 97 shows a cell in the columnar form; fig. 98 a flattened cell with, just beneath the border, a layer of granules staining quite differently from the blood-débris externally.

Figs. 99-103.—Various details of the cells after Flemming-fixation; fig. 99, red-staining grains in the upper end of the cell; fig. 100, red-staining grains mixed with osmic-blackened (fatty) grains; fig. 101, cell comparatively free from granules; fig. 102, pseudosphere in the upper end of a cell; fig. 103, two "yellow bodies" in the upper end of a cell.

Fig. 104.—Portion of the epithelium close to a crypt of regeneration, showing a recurved trypanosome attached to an epithelial cell and a trypanosome of ordinary type free in the débris close by. Twenty-four hours.

Fig. 105.—Section shaving the side of an epithelial cell obliquely, showing a trypanosome of ordinary appearance attached to it. Twenty-four hours.

Fig. 106.—Stout form of trypanosome free in the débris. Twenty-four hours.

Fig. 107.—Recurved trypanosome within a cell. Twenty-four hours.

Fig. 108.—Tailed sphere within a cell, also two smaller spheres and a portion of a trypanosome cut across; the nucleus of the cell does not come into the section. Twenty-four hours.

Fig. 109.—Portion of the epithelium showing a very large tailed sphere, three smaller ones and a nucleus (*N*); the cells are much exhausted. Twenty-four hours.

Fig. 110.—Section of a cell (not passing through the nucleus) showing a large tailed sphere and fragments of three smaller ones. Twenty-four hours.

Fig. 111.—A large tailed sphere and a slice of a smaller one, in a cell near the nucleus (*N*). Twenty-four hours.

Fig. 112.—Numerous multiplication-stages in a partly broken-down cell of flattened type, and a cell nucleus (*N*). Twenty-four hours.

Fig. 113.—Six spheres in a broken-down cell cast off into the blood-débris. Twenty-four hours.

Figs. 114-116.—Three figures drawn from the same slide. Fig. 114, exhausted cell filled with trypanosomes; fig. 115, a large sphere ripe for breaking up into a mass of trypanosomes as in the last; fig. 116, large sphere. The preparation was over-extracted and does not show the flagella. Twenty-four hours.

PLATE 39.

[Stages of the stomach-phase, all from the same series of sections through nine stomachs preserved thirty-six hours after the infective feed in Flemming's fluid and stained with iron-hæmatoxylin and Licht-grün-picric. Drawn with the camera lucida at a magnification of 2000.]

Figs. 117-129.—Extracellular trypanosomes.

Fig. 117.—Slightly club-shaped form, near the epithelium but not attached.

Figs. 118, 119.—Recurved forms, not attached.

Figs. 120-123.—Attached recurved forms,

Fig. 124.—A small bunch of trypanosomes attached in the interspace between two cells. Structure of the trypanosome difficult to make out clearly.

Fig. 125.—Trypanosomes in or attached to the débris of a necrosed cell. Two of them are recurved forms, the third possibly erithridial in type.

Fig. 126.—A slightly hypertrophied epithelial cell containing three spheres of different sizes, with portions of the cells on either side of it. *N*, the nuclei of the cells; *l.m.*, longitudinal muscles of the stomach in transverse section; *c.m.*, one of the circular muscles of the stomach.

Fig. 127.—From the next section of the series, showing the same cell in which appears one of the same three spheres lodged in a distinct vacuole.

Fig. 128.—Large hypertrophied cell with five nuclei in process of being thrown off from the epithelium; it contains two spheres (*sph.*) and numerous yellow bodies. The same cell is shown in the photograph in Pl. I. fig. 313 c.

Fig. 129.—Flattened cell showing a sphere lodged in a vacuole beside the nucleus (*N*).

Fig. 130.—Cell containing four spheres close to the nucleus (*N*).

Fig. 131.—Young epithelial cell close to a crypt containing an early multiplication-stage just external to the nucleus (*N*).

Fig. 132.—Degenerated and cast-off cell, containing a sphere, the flagellum of which is sticking out from the remains of the cell.

Fig. 133.—Recurved trypanosome within a cell.

Fig. 134.—Normal columnar epithelial cell containing spheres both above and below the nucleus (*N*).

Fig. 135.—Degenerated and cast-off epithelial cell, full of fatty deposits and yellow bodies, containing two spheres.

PLATE 40.

[Epithelial cells of the flea from sections of stomachs, all, except fig. 147, fixed with Flemming's fluid and stained with iron-hæmatoxylin followed by Lichtgrün-picric. Figs. 136-140 are drawn at a magnification of 1000; all the rest at 2000.]

Fig. 136.—Four young cells showing the partially developed border and the different positions of the nucleus in the cell.

Fig. 137.—A cell showing the more granular condition.

Figs. 138, 139.—Two stages of the fatty degeneration of the cell. In 138 the nucleus, though obscured, is still faintly visible; in 139 the cell has become an opaque black mass.

Fig. 140.—Two contiguous cells, of which the one to the left shows the beginning of the "yellow necrosis."

Figs. 141, 142.—The upper ends of cells containing "yellow bodies"; in fig. 141 several such bodies, in fig. 142 one large one.

Figs. 143, 144.—To show the deposition of osmic-blackened fatty grains in cells beginning to degenerate; fig. 143 in a flattened cell, fig. 144 in a columnar cell.

Figs. 145, 146.—Cells containing pseudospheres (*ps.*) in their upper portions.

Fig. 147.—To show the deposition of deeply-staining grains below the border of the epithelial cell. External to the cell are seen the coarse black grains of the blood-débris. Maier's fluid, iron-hæmatoxylin, Lichtgrün-picric.

Fig. 148.—Outer end of a cell containing a sphere (*sph.*) which is seen to contain a darkly-staining mass (chromatin? or fatty deposit?).

PLATE 41.

[Various stages of the rectal-phase. Figs. 201, 202 are from sections fixed with Flemming's fluid, stained with Giemsa's stain and drawn at a magnification of 2000; all the other figures are from smear-preparations fixed with osmic vapour, stained with Giemsa's stain, and drawn at a magnification of 3000.]

Figs. 149-152.—Early "tadpole" forms from the rectum, twenty-four hours; fig. 152 is perhaps an early stage of division.

Figs. 153, 154.—Early stages from the rectum, twenty-four hours.

Fig. 155.—Rectum, thirty-six hours.

Fig. 156.—Rectum, twenty-four hours.

Figs. 157, 158.—Rectum, thirty-six hours.

Figs. 159-161.—From the rectum of another flea, thirty-six hours.

Fig. 162.—Rectum, sixty hours.

Figs. 163, 164.—rectum, three days.

Figs. 165-170.—From a swarming rectal infection, three days.

Figs. 171-176.—From a swarming rectal infection, about three and a half days old.

Figs. 177, 178.—From the rectum, forty-eight hours.

Figs. 179-182.—From the rectum about three and a half days.

Figs. 183-187.—Early stages from the rectum, four days. In the clump shown in fig. 183 two trypanosomes of quite ordinary type are seen; these are probably forms which have come in with a later feed and have attached themselves secondarily to the clump.

Figs. 188-190.—From the rectum, five days.

Fig. 191.—From the rectum of another flea, five days.

* Figs. 192-200.—From the recta of fleas taken from the infective breeding-cage, age uncertain. Fig. 192, early form from the rectum; figs. 193-196, from stomach-preparations (post-pyloric?); fig. 197, rectum; fig. 198, rectum; fig. 199, stomach (post-pyloric); fig. 200, rectum.

Fig. 201.—Free clump from a section of a rectum.

Fig. 202.—From a section of the intestine close behind the pylorus, showing a number of haptomonads attached to the cuticle, and between them, projecting up above the level of the haptomonads and distinguished from them by their lighter stain and terminal *nn* are numerous examples of the final trypanosome-forms (*T*).

PLATE 42.

[Various stages of the rectal-phase. Figs. 273-277 are from sections of recta fixed with Flemming's fluid, stained with iron-hæmatoxylin, and drawn at a magnification of 2000; all the other figures are from smear-preparations fixed with sublimate mixtures, stained with iron-hæmatoxylin and drawn at a magnification of 3000.]

Figs. 203, 204.—Forms from the intestine in process of migration to the rectum, twenty-four hours (see p. 568).

Figs. 205-212.—Early "tadpole" forms from the rectum, twenty-four hours.

Figs. 213, 214.—Early division-forms, rectum, forty-eight hours.

Figs. 215, 216.—Early forms, one dividing, rectum, three days.

Fig. 216 a.—Couple produced by an early division?

Figs. 217-220.—From a well-infected rectum, about three and a half days.

Figs. 221-240.—Various forms from a swarming rectal infection of a flea that had been on an infected rat for five days; figs. 221-225, haptomonads; figs. 226-230, division-forms; figs. 231-234, growth of flagellum; figs. 235, 236, nectomonads; figs. 237, 238, transitional forms; figs. 239, 240, slender and stout trypanosome-forms.

Figs. 241, 259.—Various forms from a swarming rectal infection, eight days; figs. 241-245, rounded haptomonads without free flagella; figs. 246, 250, 251, flagella growing out from rounded haptomonads; figs. 247, 248, 249, pear-shaped haptomonads without free flagella; fig. 252, clump of pear-shaped haptomonads, some with well-developed

flagella; fig. 253, division-stage; fig. 254, nectomonad; figs. 255-258 transitional forms; figs. 259, final trypanosome-form.

Figs. 260-264.—Various forms from a swarming rectal infection, taken from the infected breeding-cage, age unknown; figs. 260 a-263 a, the same specimens as 260-263, restained in Twort's stain; fig. 264, a nectomonad stained with Twort's stain. (N.B.—Figs. 260 a-263 a and 264 have been transferred to Pl. 38.)

Figs. 265-272.—Various forms from a swarming rectal infection, age unknown; fig. 265, early form? fig. 266, division-form; fig. 267, rounded haptomonad, without flagellum, beginning to divide; figs. 268, 269, rounded haptomonads with free flagellum beginning to grow out; fig. 270, transitional form; figs. 271, 272, final trypanosome-forms.

Fig. 273.—Clump of crithidial forms attached in the intestine just behind the pylorus, in sections of a stomach of a flea preserved thirty-six hours after the infective feed. ($\times 2000$.)

Figs. 274-276.—From sections through the rectum of a flea preserved eight days after the infective feed. Fig. 274, free clump; fig. 275, clump attached to the cuticle of the rectum; fig. 276, forms attached singly to the cuticle. ($\times 2000$.)

Fig. 277.—Haptomonads attached to the cuticle of the rectum, from sections through the rectum of a flea, preserved eleven days after the infective feed. ($\times 2000$.) (This drawing is from the same section as that photographed in Pl. 44, fig. 317; the patch drawn lies just to the right hand of the middle of the three pointers that start from *c* in fig. 317.)

Figs. 278-288.—*Leptomonas pattoni*, various forms from the rectum of the flea. Figs. 278-284 a are from preparations fixed with Maier's fluid and stained with iron-haematoxylin; figs. 285-288 are from preparations fixed with osmic vapour and stained with Giemsa. In the specimens drawn in figs. 281, 282, note small bodies marked *x* which appear to be endogenous buds ("infective granules"); in fig. 283 a similar body is shown free, and figs. 284, 284 a appear to be stages in the development of the bud into the leptomonad. Fig. 285 is evidently a nectomonad form, and fig. 286 a stage in the development of such a form; they differ from the nectomonads of *T. lewisi* in the great prolongation of the body behind *N*.

PLATE 43.

[Degenerative forms, fixed with osmic vapour and stained with Giemsa, drawn with the camera lucida to a magnification of 3000.]

Figs. 289, 290.—Six hours, rectum.

Figs. 291-296.—Twelve hours, rectum.

Figs. 297, 298.—Recurved forms from the rectum, twelve hours.

Figs. 299-301.—From the rectum of another flea, twelve hours.

Figs. 302-304.—Rectum, thirty-six hours.

Fig. 305.—Stomach, forty-eight hours.

Fig. 306.—Rectum, twenty-four hours.

Fig. 307.—Rectum, sixty hours.

Fig. 308.—Clump of degenerative forms, rectum, eighteen hours.

Figs. 309, 310.—Agglomerating trypanosomes from a flea fed for the second time on an infected rat the day previously.

PLATE 44.

Fig. 311.—Clump of degenerative forms (compare fig. 308). Photo. ($\times 1500$.)

Fig. 312.—Clump of developmental crithidial forms; at *T* is seen a final trypanosome-form attached to the clump. From the same preparation as figs. 241-259. Photo. ($\times 2000$.)

Fig. 313.—Infected patch of stomach-epithelium in which the cells are breaking down and being thrown off. The large multinucleate cell marked *C* is the cell, part of which is drawn in Pl. 39, fig. 128. Photo. ($\times 600$.)

Fig. 314.—Section through an epithelial crypt of regeneration. Close beside it is seen a black, degenerated epithelial cell. Photo. ($\times 800$.)

Fig. 315.—Section of an infected patch of the stomach-epithelium showing the cells being thrown off; the cells contain coarse black (fatty) granules and stages of intracellular multiplication, which are not clearly seen. Photo. ($\times 600$.)

Fig. 316.—Section of the stomach-epithelium showing an epithelial crypt towards the middle; to the left of the crypt the epithelium is old and degenerate and full of blackened fat; to the right of the crypt is new, clear epithelium budded off from it. Compare Text-fig. 2. Photo. $\times 300$.

Fig. 317.—Section of the wall of a rectum showing a swarming crithidial infection of the "pile-carpet" type. Fig. 277 is drawn from this section, from the part between the middle and the right-hand pointers which start from *c* to indicate the serried ranks of the crithidial forms. Photo. ($\times 500$.)

Figs. 318.—A sphere (the same that is drawn in fig. 96) and near it one of the long free trypanosomes of the stomach-type, from a smear. Photo. ($\times 1000$.)

Fig. 319.—Section passing through the opening of the pylorus into the intestine. Photo. ($\times 300$.)

PLATE 45.

[General diagram of the entire life-cycle of *Trypanosoma lewisi* in the flea, combined from the observations and experiments set forth in this memoir in order to give a summarised idea of the complete course of events. The stomach-phase is represented on the upper side of the diagram, the rectal phase on the lower side and to the left; in the right-hand lower corner is shown the secondary infection of the pylorus. Magnification 2000.]

1. Trypanosome as taken up by the flea from the rat.
2. Trypanosome slightly modified after a few hours in the flea's stomach.
- 3-12. Cells of the epithelium of the stomach containing the various phases of the intracellular multiplication.
3. Cell with two trypanosomes attached to it, one of ordinary, the other of recurved, type.
4. Penetration of an epithelial cell by a trypanosome, drawn from Nöller's description of the process.
5. Recurved form in the cell.
6. Rolled-up form in the cell.
7. Early multiplication-form.
8. Later multiplication; 8 *nn*, 8 *NN*.
9. Large tailed sphere.
10. Large ripe sphere without tail.
11. Daughter-trypanosomes free in the exhausted cell after bursting of the sphere.
12. Daughter-trypanosomes escaping from the cell. After being set free, the trypanosomes may do one of two things as shown by the arrows. They may each penetrate another epithelial cell and repeat the process of multiplication by which they were produced. Or they may pass through the pylorus and down the intestine to the rectum to give rise there to the rectal (erithidial) phase.
- 13-18. To show the two possible ways in which the established rectal phase may arise from the stomach-trypanosomes :
 - (a) 13, 14, 15, 16. Four successive stages of the contraction of a stomach-trypanosome into a pear-shaped form which at 17 divides by

equal or subequal binary fission to produce two equipotential daughter-products, similar to 18, which by further-repeated binary fission produce the ordinary crithidial phase.

(b) 14a, 14b. Two successive stages of the contraction of a stomach-trypanosome into a club-shaped form, which at 17a divides by unequal binary fission to produce two inequipotential products, the larger parent-form which does not develop further and the smaller daughter-form, similar to 18, which by repeated binary fission produces the ordinary crithidial phase.

19. Established rectal infection, showing the various forms of the rectal phase. *h.h.* Haptomonads, some of them dividing, some of them transitional to the other types. *n.n.* Nectomonads. *tr. tr.* Forms transitional from the crithidial type to *T.T.*, the final trypanosome-form.

The final trypanosome-forms, as shown by the arrows, pass finally out of the flea with the faeces.

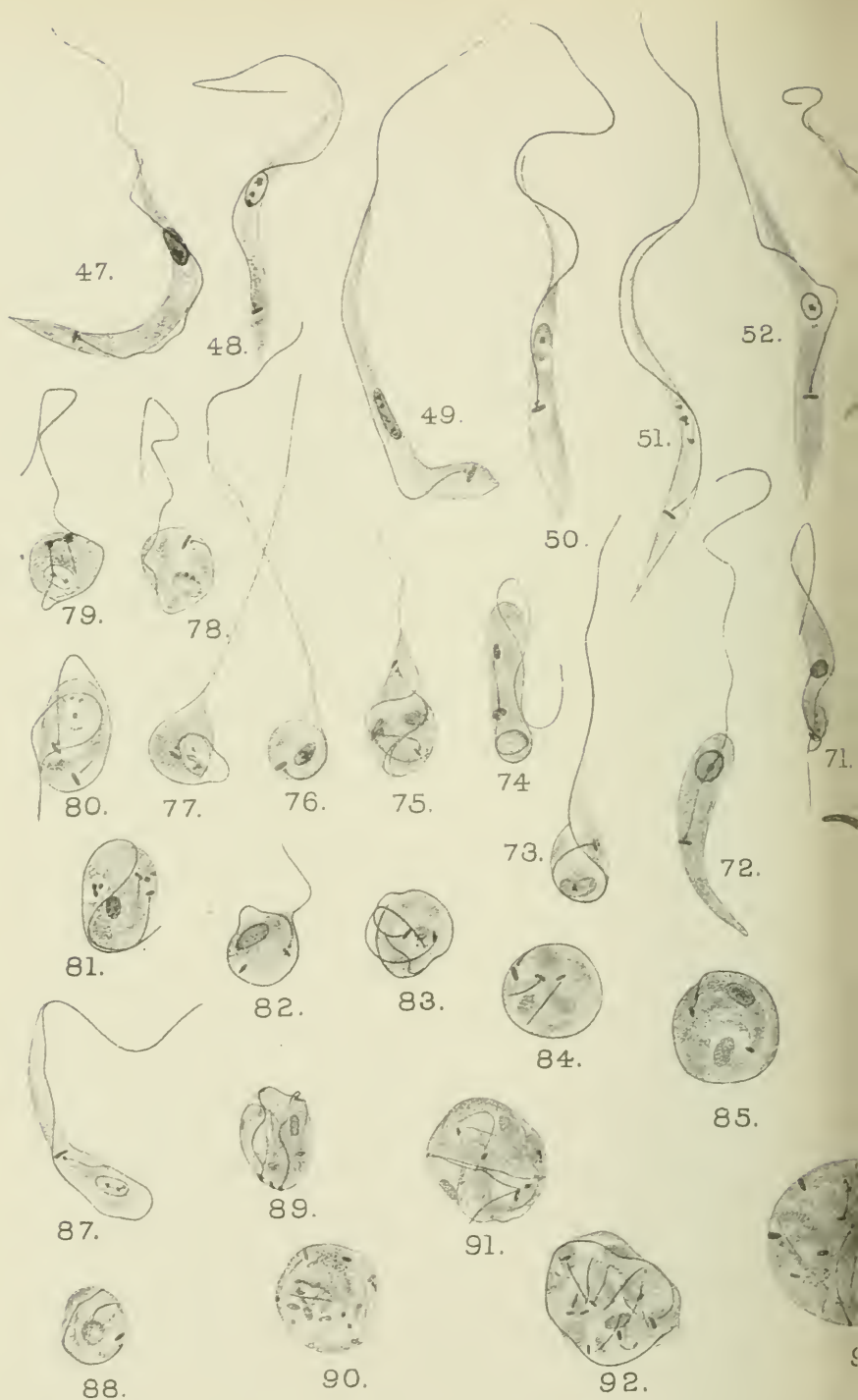
The nectomonads can, under certain conditions of food-supply, migrate back along the intestine and fix themselves close behind the pylorus in order to give rise to—

20. Secondary infection of the pyloric region of the intestine: the letters have the same significance as before. Final trypanosome-forms (*T.T.*) arise and pass down the intestine into the rectum and so out with the faeces; nectomonads (*nn*) also arise and may migrate back again to the rectum and re-establish the crithidial phase there; these migrations are indicated by the arrows.









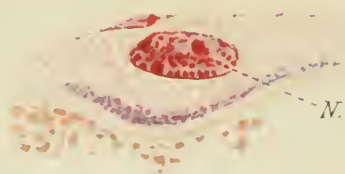


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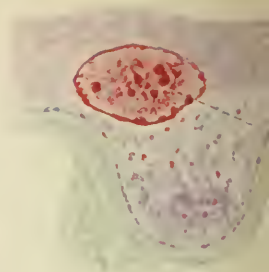




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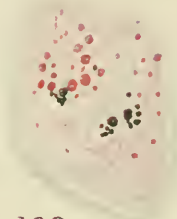
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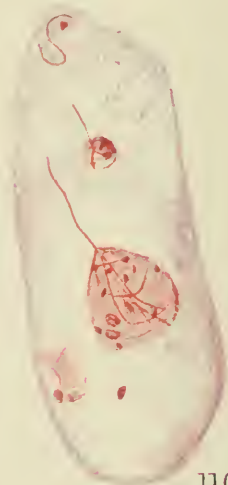
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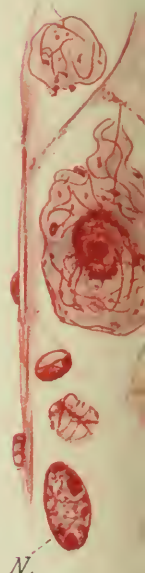
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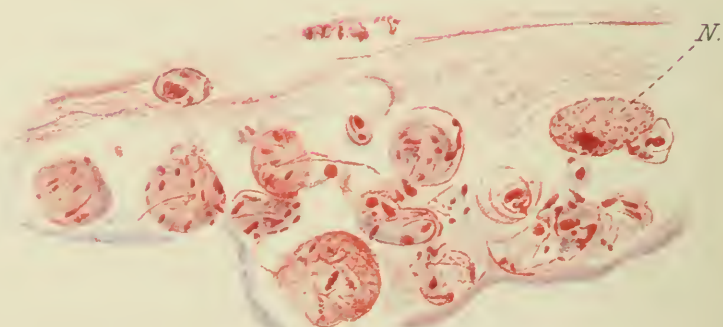
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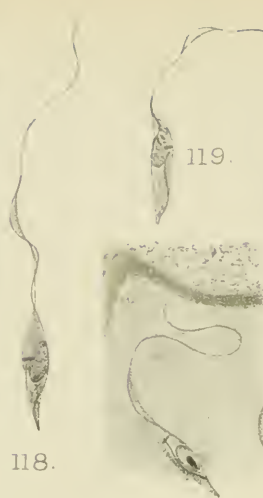
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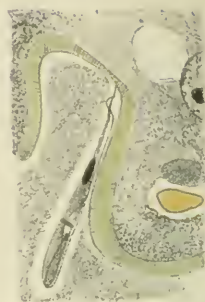


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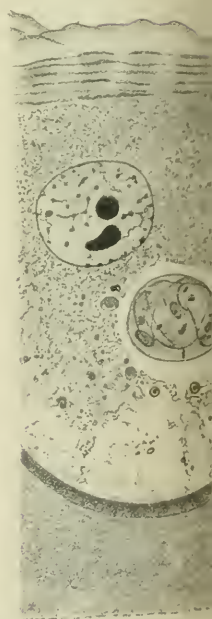


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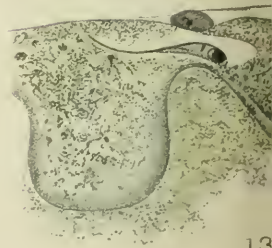


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Hutch Lith^r London



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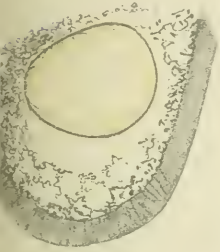
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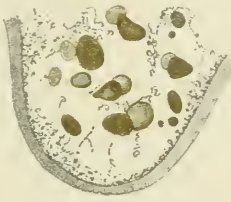
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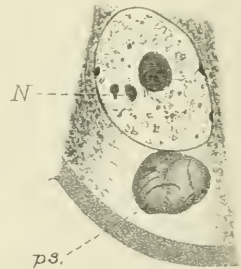
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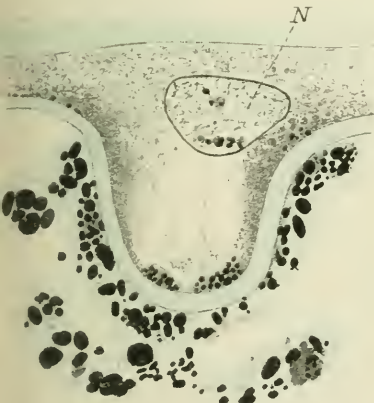
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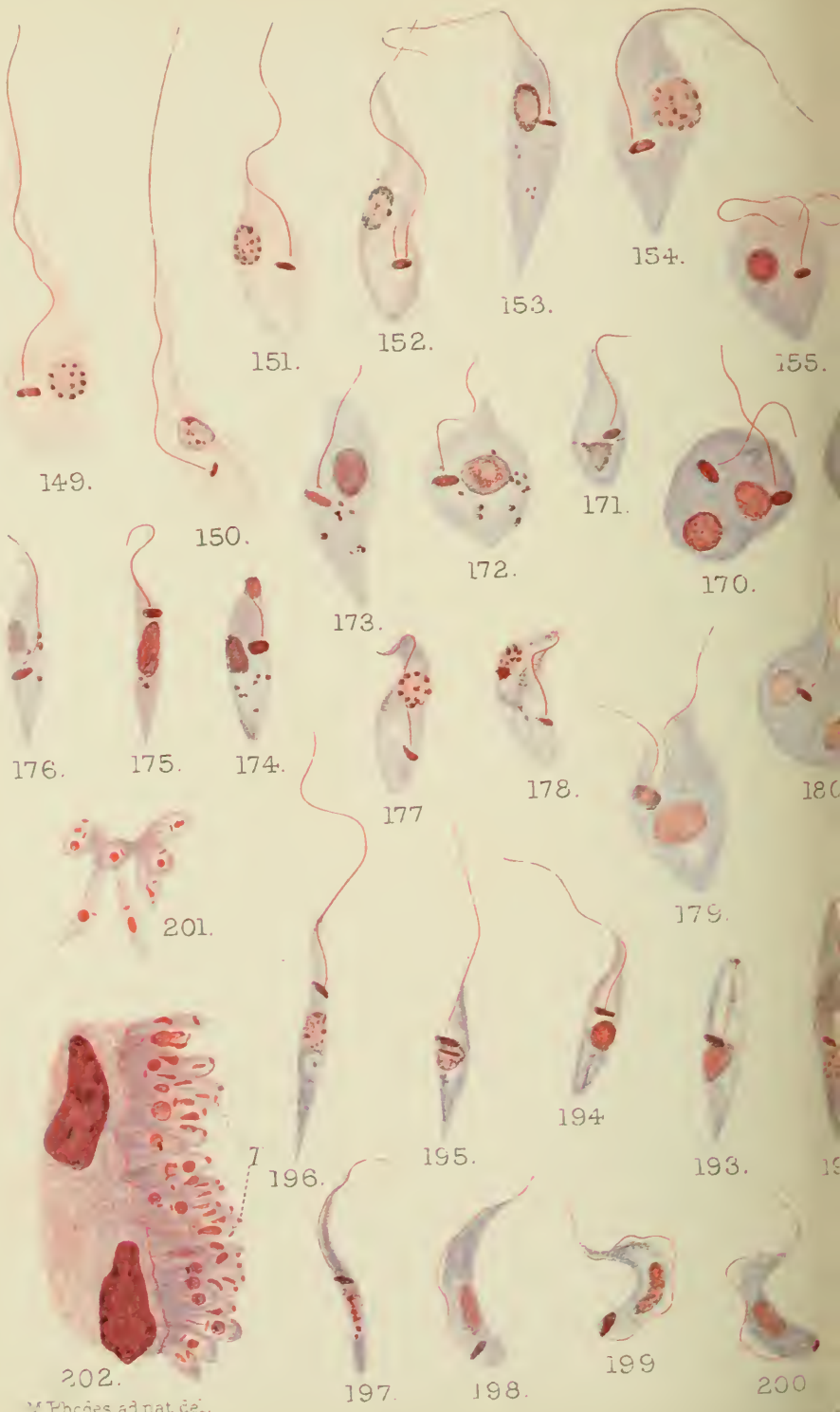
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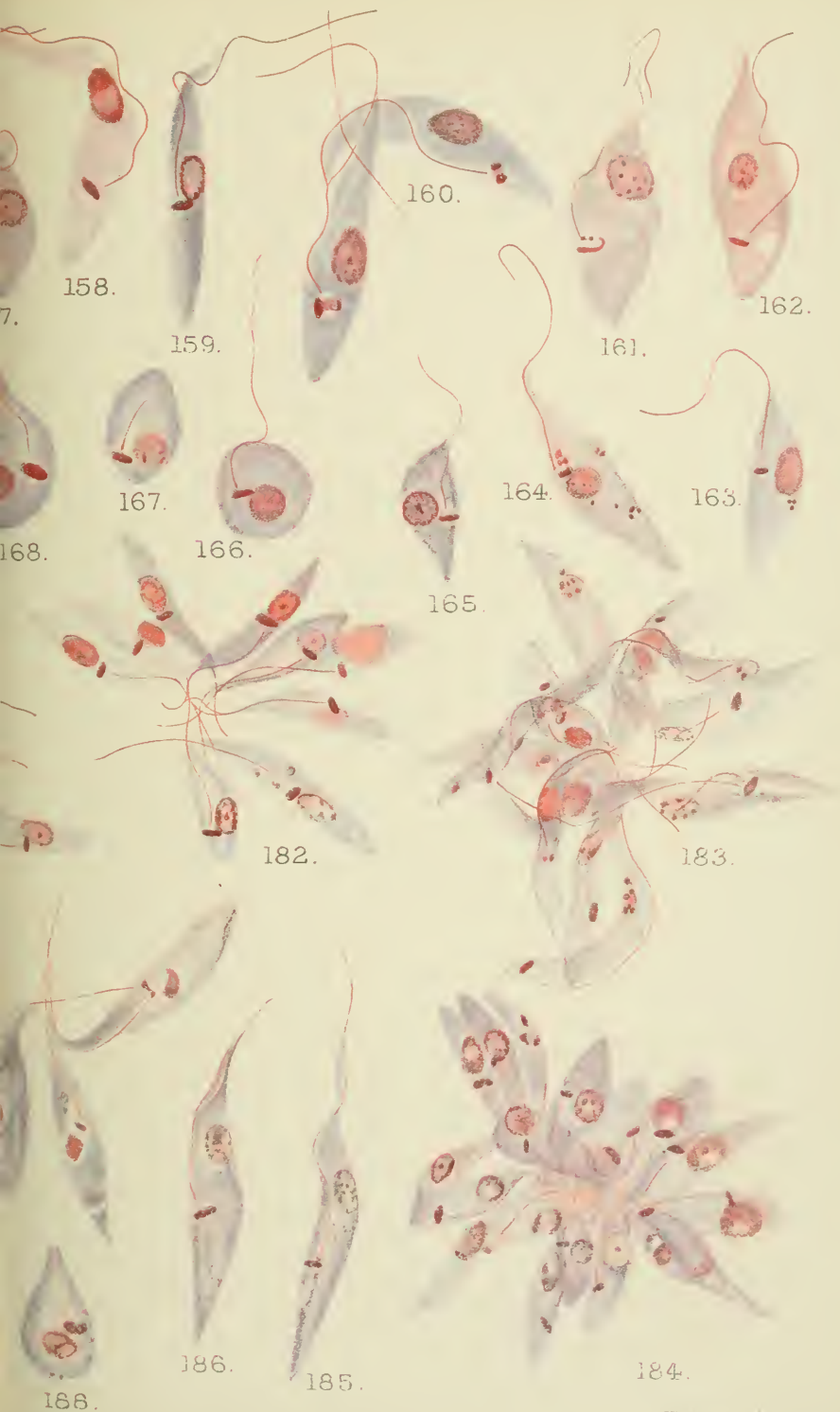
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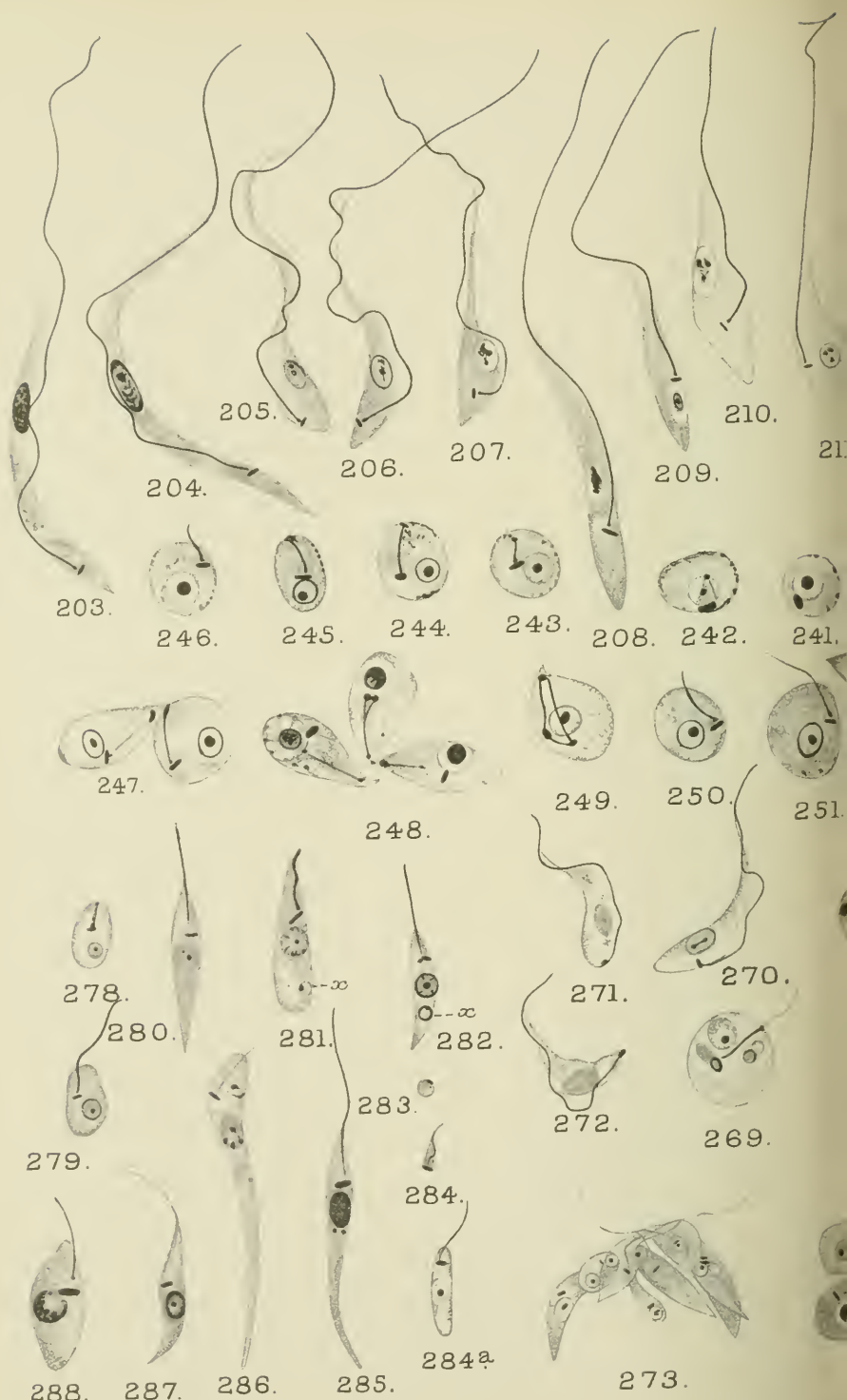
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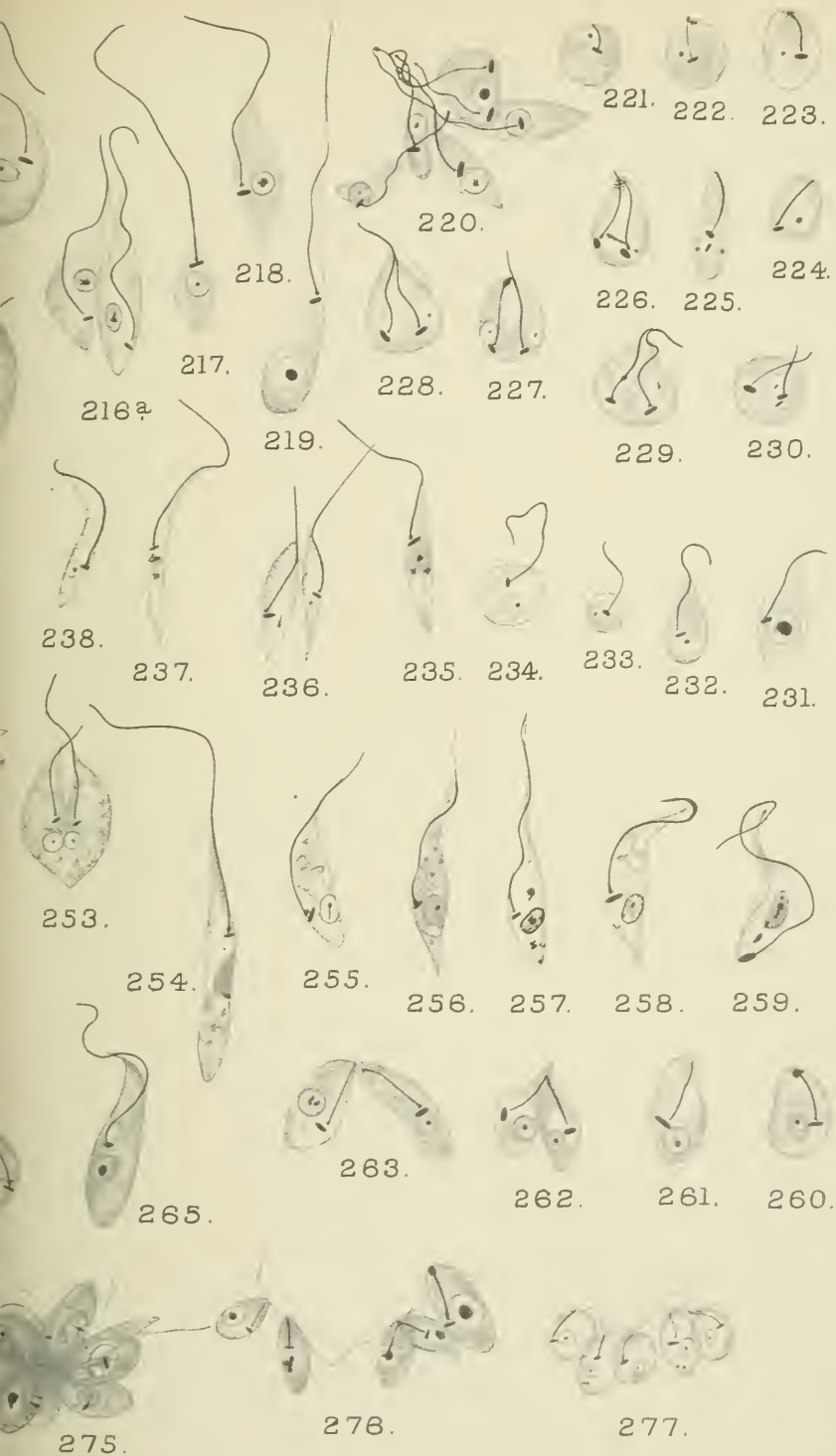
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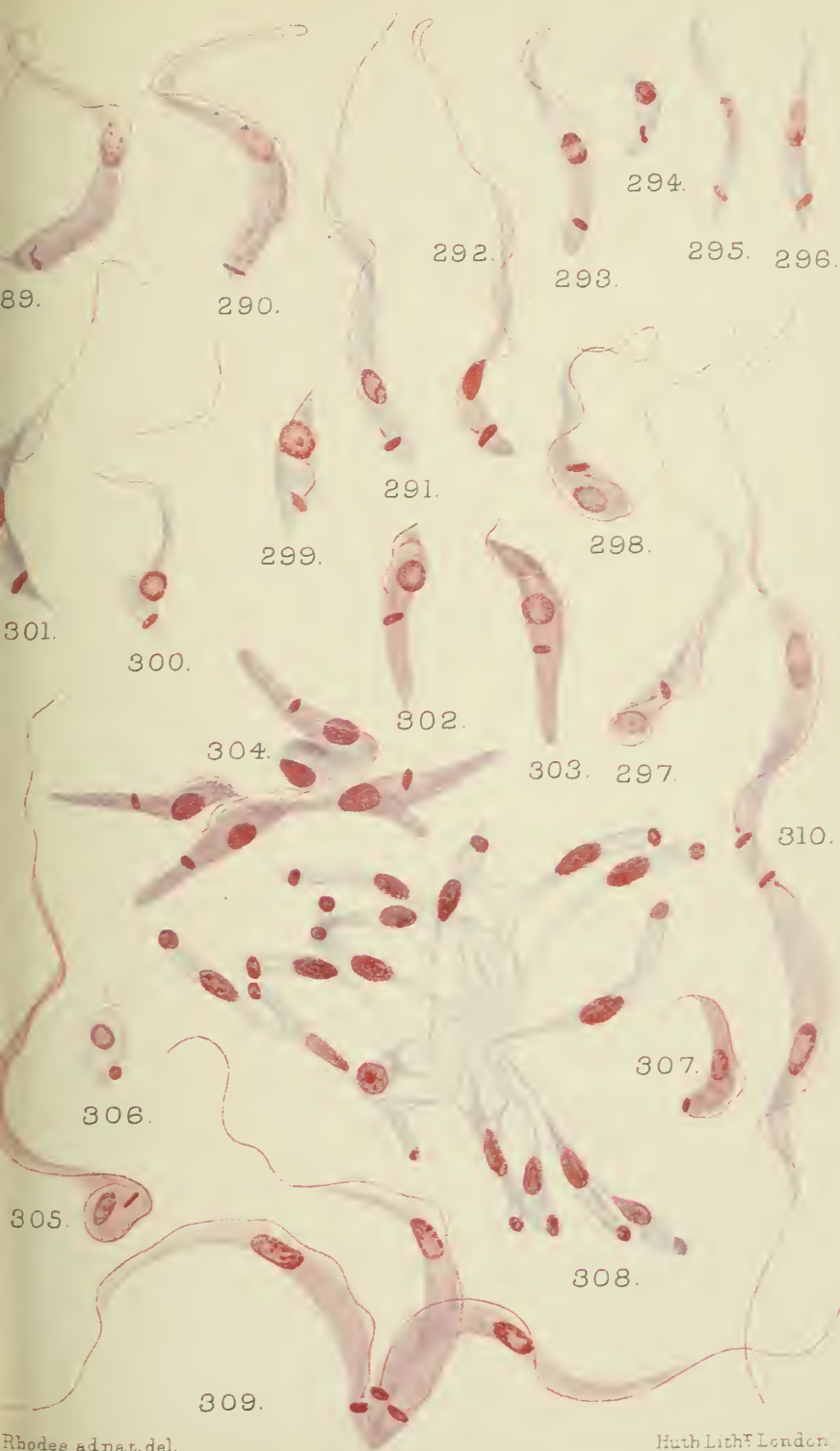
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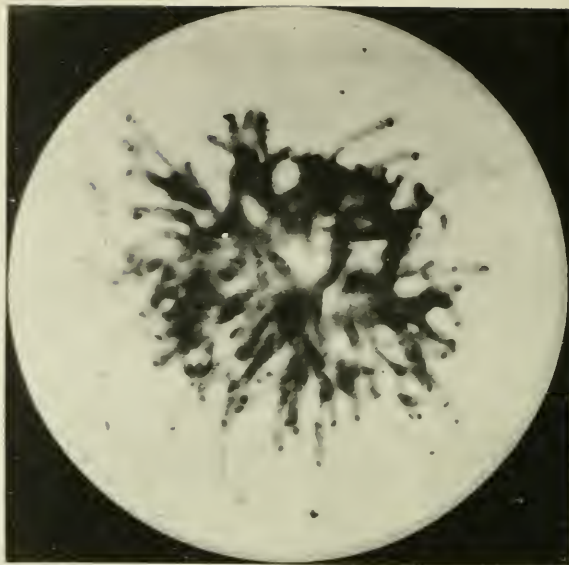
ATTONI IN THE FLEA—RECTAL PHASE.

For Figs. 240^a, 241^a, 242^a, 243^a, 244^a, see Pl. 38.

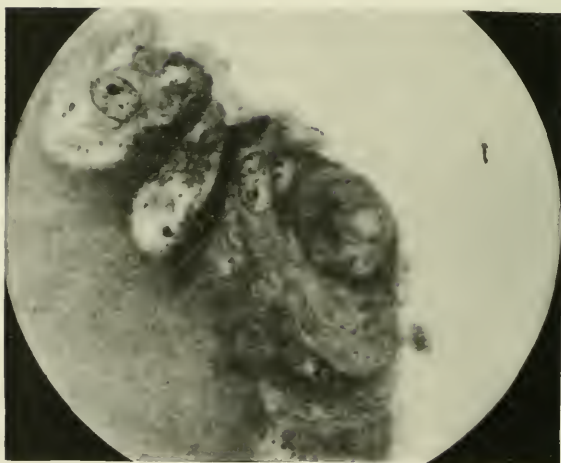


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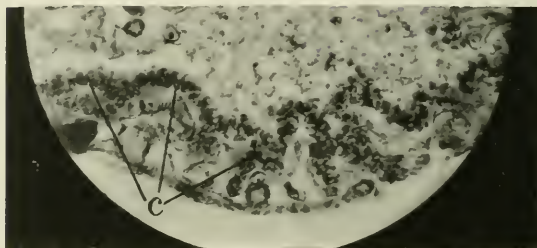
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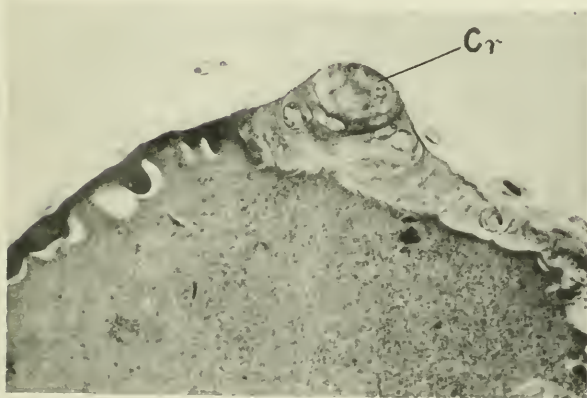
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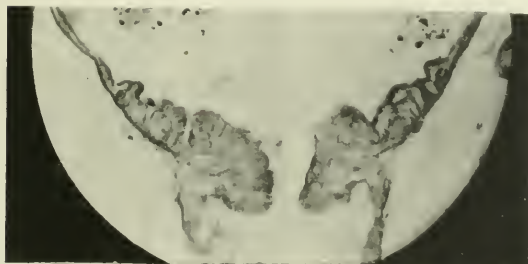
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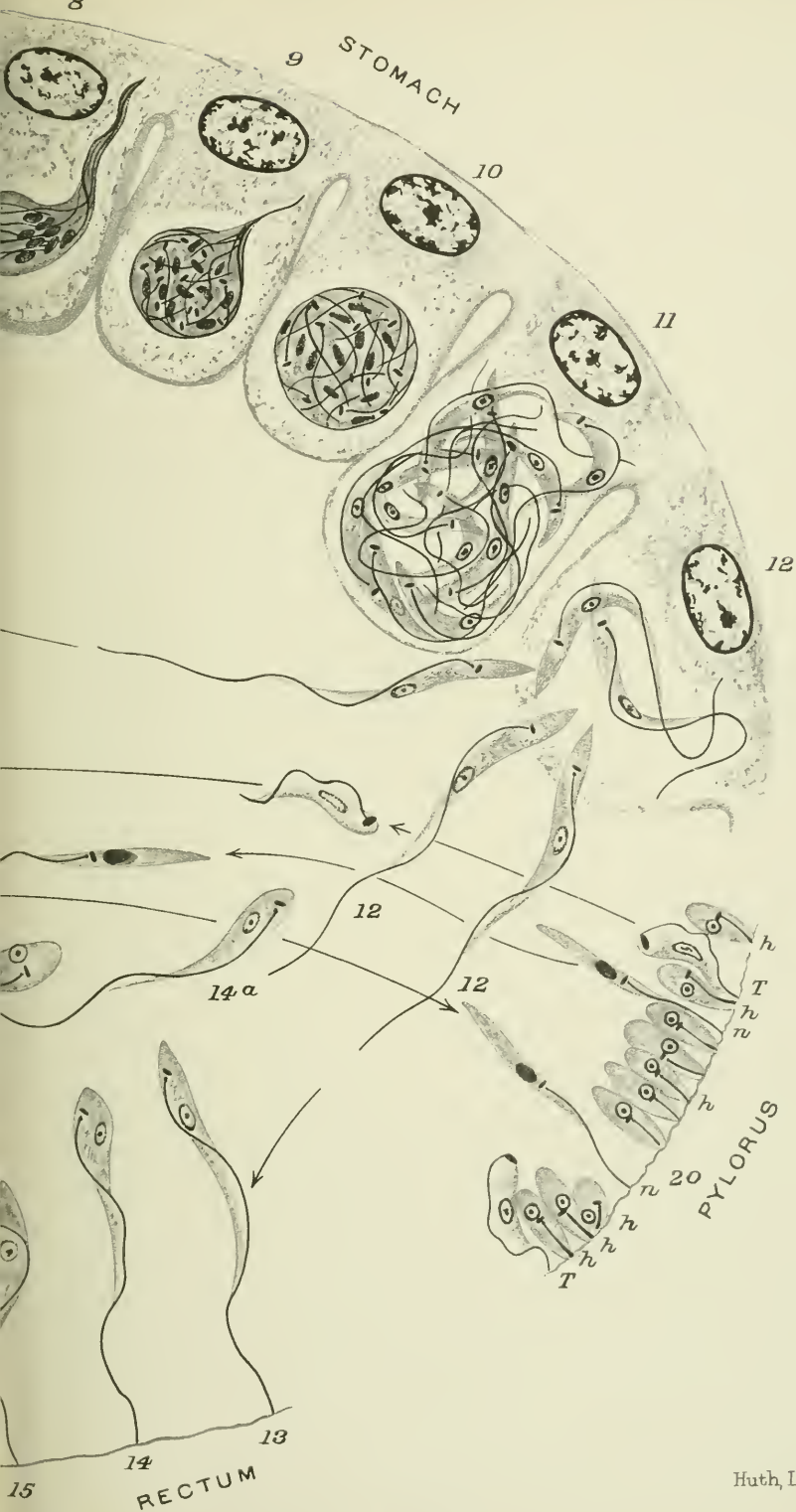


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Huth, London.

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11-47

XLVI. THE HYDROLYSIS OF GLYCOGEN BY DIASTATIC ENZYMES. Part III. FACTORS INFLUENCING THE END-POINT OF THE HYDROLYSIS.

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(*Received July 14th, 1914.*)

In a previous communication [Norris, 1913, 1] the author confirmed the result previously obtained by Philoche [1908] that in the hydrolysis of glycogen by diastatic enzymes, the hydrolysis is as a rule far from complete, and also pointed out that the final degree of hydrolysis, as represented by the maltose production, increases with rising concentrations of enzyme though at a very much slower rate. This is therefore rather a different case from the breaking down of starch, where, although the hydrolysis is not complete, the equilibrium point according to most workers is the same with different concentrations of enzyme, viz. about 80·8 per cent. maltose and 19·2 per cent. dextrin. In this connection however Maquenne and Roux [1906, 1, 2, 3] suggest that the reaction only comes to an end owing to an insufficient activity of the enzyme and state that if the diastase be activated by the addition of traces of acid the hydrolysis proceeds to completion, a result which has been confirmed by Bayliss [1914].

It was also found by Brown [1904] that if the maltose formed were removed by fermentation with yeast the whole of the dextrin was hydrolysed, a result which suggested that the products exerted a retarding influence. As however acid is produced from the yeast this result may be really due to the activation of the enzyme and not to the removal of the products.

Under these circumstances it seemed of interest to examine the causes limiting the hydrolysis of glycogen by the glycogenase of the pancreas and some experiments carried out from this point of view are described in the present communication.

The glycogen used was prepared from oysters by Pflüger's method and

purified by repeated precipitation with alcohol. In those experiments where the influence of salts was under investigation the solutions were dialysed before use for several days against frequent changes of distilled water in presence of toluene.

The enzyme was as a rule prepared from dried pigs' pancreas which had previously been rendered fat free, by extraction with 50 per cent. alcohol and precipitation of the filtered extract with alcohol and ether. The precipitate was again taken up in 50 per cent. alcohol, filtered and reprecipitated, this process being repeated two or three times.

The course of the hydrolysis was followed by estimations from time to time of the reducing power of the solutions by Bertrand's method, the assumption being made that the reduction was due to maltose alone. While this is probably not entirely correct, the method is far more convenient than estimations of the optical rotation. The latter is open to the same objection as the former method and in addition necessitates the employment of very dilute solutions whereby further inaccuracies are in all probability introduced. In many cases the iodine reaction was also followed during the experiment.

Exp. 1. Influence of enzyme concentration on the hydrolysis of glycogen.

A. 105.5 cc. solution containing 1 g. glycogen, 0.1 cc. enzyme and 1 cc. toluene.

B. 105.5 cc. solution containing 1 g. glycogen, 0.5 cc. enzyme and 1 cc. toluene.

C. 105.5 cc. solution containing 1 g. glycogen, 5.0 cc. enzyme and 1 cc. toluene.

The above were incubated at 37° and samples taken from time to time for analysis, the results being shown in the following table.

TABLE I.

Duration of experiment Hours	Percentage hydrolysis		
	A.	B.	C.
1	36.65	52.90	69.8
2	40.45	54.00	77.1
3	42.80	54.30	82.0
4	43.70	54.55	84.8
5	44.60	54.70	87.4
6	45.10	—	89.0
7	45.40	—	90.2
8	45.60	—	91.0
9	45.60	55.10	91.5

In the above experiment it will be seen that while with the highest concentration of enzyme rather over 90 per cent. of the glycogen was completely hydrolysed to maltose, with 0.1 cc. enzyme only about half this quantity of maltose was formed. At the same time all the glycogen had disappeared, having been converted into a dextrin which remained to a large extent unhydrolysed. The intermediate products in the hydrolysis of glycogen have not received a great amount of attention. Tebb [1898] however found that a dextrin was formed which was extremely resistant to further hydrolysis and identified this with the dextrin previously described by Seegen [1879] and termed by him dystropodextrin. This dextrin gives no iodine reaction, in fact with pancreatic glycogenase no erythrodestrins appear to be formed, or if they are produced they are hydrolysed so rapidly that it is impossible to detect them.

It is difficult to believe however that the resistant nature of this dextrin is the cause of the stopping of the hydrolysis. For considering the experiment just described, the rates fell off when the reduction indicated a hydrolysis of rather over 40 per cent. in *A*, of 50 per cent. in *B*, and over 80 per cent. in *C*. In other words the slower the initial rate of hydrolysis, the greater was the quantity of resistant dextrin apparently formed. In the case of *C* the slowing off in the rate could be quite well explained by the exhaustion of the substrate, but in *A* and *B* this explanation cannot hold. Another possible solution is that two enzymes may be concerned. This view in the case of diastase has been advanced by several investigators [Brown and Heron 1879; Kjeldahl 1879; Duclaux 1899; Fraenkel and Hamburg 1906]. Now if the enzyme preparation employed in these experiments consists of a large proportion of glycogenase which only carries the hydrolysis to the dextrin stage and a very small amount of dextrinase the results obtained above are to a certain extent what would be expected. For in this case with the low enzyme concentration there might be enough glycogenase to carry out the first stage of the hydrolysis but insufficient dextrinase to hydrolyse the dextrin thus formed at a corresponding rate. In this way an accumulation of dextrin would take place. With the high concentration of enzyme the rate of the first stage of the hydrolysis would be limited by the concentration of glycogen employed and hence would not increase proportionally with rising enzyme concentrations. The second stage of the hydrolysis however would proceed at a much greater rate and hence the accumulation of dextrin would be less as the concentration of enzyme increased. Now while this is actually the final result found experimentally an examination of Table I shows that

when the slow stage of the hydrolysis is reached, *B* and *C* are proceeding not very much more quickly than *A*. Hence some other factors must be concerned and the two which first suggest themselves are (1) destruction of the enzyme during the course of the experiment, and (2) retarding influence of the products. Before considering these points however it will be as well to describe some further experiments in which the results indicated above were reproduced by other methods.

It is well known that the activity of a glycogenase preparation is almost completely lost on dialysis but can be restored by the addition of certain salts, notably chlorides. A certain minimum concentration of such salts varying with the concentration of enzyme is required to produce the optimum rate of hydrolysis [Cole 1906; Starkenstein 1910, 1; Norris 1913, 2].

Now if a series of dialysed glycogen solutions is made up, each containing the same quantity of dialysed glycogenase but increasing quantities of KCl, not only does the initial rate of hydrolysis increase with rising concentration of salt but the final degree of hydrolysis also rises. These results only obtain of course until the salt concentration reaches the optimum required for the particular strength of enzyme employed.

Exp. 2. Influence of salt content on the total degree of hydrolysis.

A. 100 cc. 1 per cent. dialysed glycogen containing 1 cc. toluene and 0.5 cc. dialysed enzyme.

B. 100 cc. 1 per cent. dialysed glycogen containing 1 cc. toluene, 0.5 cc. N/20 KCl and 0.5 cc. dialysed enzyme.

C. 100 cc. 1 per cent. dialysed glycogen containing 1 cc. toluene and 2.0 cc. N/20 KCl and 0.5 cc. dialysed enzyme.

The solutions were incubated at 37° and the reducing power estimated at frequent intervals.

In this experiment *A* which contained no added salt had still a low activity, the dialysis not having been carried sufficiently far to render the solution entirely salt free. *A* therefore represents a solution with very low salt content.

Results :

TABLE II.

Duration of experiment Minutes	Percentage hydrolysis		
	A.	B.	C.
15	2.07	9.2	13.2
30	3.22	14.6	22.7
60	5.07	21.6	—
75	—	—	30.18
120	10.14	25.68	—
135	—	—	33.71
240	12.89	28.4	—
255	—	—	37.44
360	—	29.28	—
420	16.58	—	38.34
480	—	29.73	39.24

The results are therefore exactly similar to those obtained when different concentrations of enzyme are employed and indicate that a certain amount of the enzyme remains entirely inactive in presence of insufficient salt. This is of some interest as Starkenstein [1910, 2] has shown that even in the absence of any salt, adsorption of diastase by starch still takes place though no chemical change results until salt is added. The salt presumably must have some specific activating effect on the enzyme and its only function cannot be to assist the combination between enzyme and substrate. It also shows that if two enzymes are present they are equally dependent on the presence of salts.

In view of the above experiment it was of interest to see what would be the effect of adding further salt to a solution such as *A* or *B* above when the rate of hydrolysis had become stationary and hence the following experiment was carried out.

Exp. 3. Addition of salt at "stationary point."

A. 50 cc. 2 per cent. glycogen + 0.25 cc. N/20 KCl + 1 cc. toluene + 0.5 cc. enzyme + 48.25 cc. H₂O.

B. 50 cc. 2 per cent. glycogen + 10 cc. N/20 KCl + 1 cc. toluene + 0.5 cc. enzyme + 38.5 cc. H₂O.

C. As *A*.

The glycogen and enzyme solutions were dialysed before use. When the rate of hydrolysis in *A* and therefore in *C* had become extremely slow, further salt was added to *C* so that the concentration of KCl became equal to that in *B*.

The course of the hydrolysis in each case is shown in Table III below.

TABLE III.

Duration of experiment Minutes	Percentage hydrolysis		
	A. Low salt content	B. High salt content	C. Low salt con- tent. Salt added at stationary point
15	3.95	10.14	—
30	7.39	17.40	7.39
240	24.80	33.50	24.90
315	26.50	35.20	26.54
			KCl added here
345	26.80	35.80	28.60
405	27.50	—	31.40
465	27.70	—	32.32
585	28.30	37.80	34.16
705	28.78	38.20	34.63

The results show that the addition of salt to *C* after 315 minutes resulted in an increase in the rate of hydrolysis, *C* increasing 8.1 per cent. during the remainder of the experiment as against the 2.2 per cent. increase shown by *A*. Compared with *B* however, which contained the high salt concentration all the time, *C* still showed a deficit of 3.6 per cent. when the two rates had become equal. This however would be expected as even assuming that all the excess of enzyme not combined with salt were intact when further salt was added, there was at this point already present in *C* a considerable percentage of the hydrolytic products which might retard the freshly activated enzyme. At the same time if the curves shown in Fig. 1 representing this experiment be observed it will be noticed that the rate of action in *C* after the addition of the salt was slightly less than that obtaining in *B* when at the same stage of the hydrolysis, this suggesting that slight injury to the enzyme had occurred.

The case when further enzyme is added at the stationary point is also of interest and will be considered next.

Exp. 4. Addition of enzyme at "stationary point."

A. 50 cc. 2 per cent. dialysed glycogen + 0.25 cc. N/20 KCl + 1 cc. toluene + 0.5 cc. dialysed enzyme.

D. 50 cc. dialysed 2 per cent. glycogen + 10 cc. N/20 KCl + 1 cc. toluene + 2 cc. dialysed enzyme.

E. 50 cc. 2 per cent. dialysed glycogen + 0.25 cc. N/20 KCl + 1 cc. toluene + 2 cc. enzyme.

F. As *A*. Enzyme added at stationary point so that enzyme concentration then became equal to *E*.

G. As *A.* Enzyme and salt added at stationary point so that the mixture was then similar in composition to *D.*

The hydrolyses were carried out in the usual manner till *F* and *G* had become very slow, which occurred after about five hours, when the additions were made, the results being shown in Table IV.

TABLE IV.

Duration of experiment minutes	Percentage hydrolysis				
	<i>A.</i> Low salt, low enzyme content	<i>D.</i> High salt, high enzyme content	<i>E.</i> Low salt, high enzyme content	<i>F.</i> Addition of enzyme	<i>G.</i> Addition of enzyme + salt
300	26.2	42.1	36.0	26.2	26.2
315	26.5	42.4	36.2	26.54	Enzyme and salt added
330	26.65	42.6	36.3	—	32.32
345	26.80	42.8	36.4	29.36	—
390	27.30	—	—	—	37.90
405	27.50	—	—	31.86	38.50
585	28.30	43.15	37.8	35.11	41.60
705	28.78	43.69	38.3	35.60	42.05

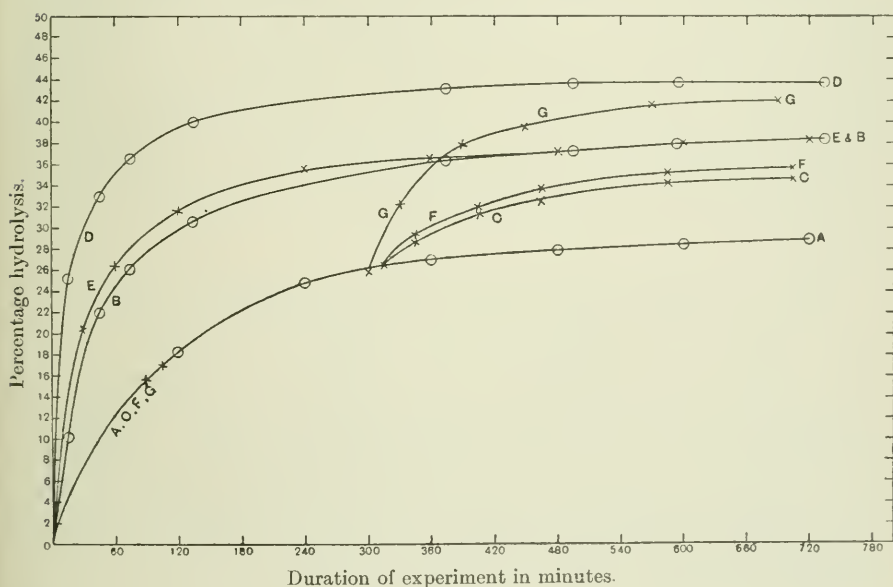


Fig. 1.

The first point of interest is that addition of further enzyme to *F* produced an acceleration although the concentration of salt employed was quite insufficient to permit the enzyme initially present to produce its maximum

effect. There is evidently then an equilibrium established between free salt, free enzyme and the salt-enzyme complex which is changed by the addition of further enzyme, more of the complex being formed and the hydrolysis thereby accelerated. The same point is indicated by the two controls *A* and *E*, *E* proceeding at a much greater rate than *A* although having the same low salt content. The results are shown graphically in Fig. 1, together with those of Experiment 3.

The addition of salt and enzyme naturally produces a greater acceleration than the addition of either separately, and this acceleration is in fact greater than the sum of the two components as one would expect, the enzyme in *F* being retarded by an insufficiency of salt. At the same time the rate in *G* after the addition is less than that obtaining in the control *D* when at the same stage of the hydrolysis. Examining the total degree of hydrolysis produced it will be seen that while *G* never quite reached the control *D*, it approached it very nearly and this also applies to *F* and its control *E*, the deficiency being however slightly greater in this case.

All these results are against the view that the stopping of the action is caused by the resistance of the residual dextrin to further hydrolysis, the addition of further enzyme producing initially a fairly rapid rate of hydrolysis which quickly falls off again, thus suggesting an alteration of an equilibrium between enzyme and the hydrolytic products. The only other obvious factor is the possible destruction of the enzyme during the course of the experiment and the degree to which this is concerned was examined in the following experiment.

Exp. 5. Addition of further glycogen at the "stationary point."

- A.* 100 cc. 1 per cent. glycogen + 1 cc. toluene + 1 cc. enzyme.
- B.* 100 cc. 2 per cent. glycogen + 1 cc. toluene + 1 cc. enzyme.
- C.* Initially as *A*. Further glycogen added after 4 hours hydrolysis.

Hydrolysis was allowed to proceed at 37° until the rate in *A* and *C* became slow and then further glycogen was added to *C* so that the total concentration became equal to that originally present in *B*, and the further course of the hydrolysis observed.

The results are shown graphically in Fig. 2, the abscissae representing the duration of the experiment in minutes and the ordinates the amount of hydrolysis which had taken place expressed in percentages of one gram, the latter being calculated as previously explained on the assumption that the reducing power of the solution is due to maltose alone.

The glycogen was added to *C* at 240 minutes and as will be observed was rapidly hydrolysed, showing that a large proportion of the enzyme was still in an active condition although the rate was much lower than that at which the first quantity of glycogen was initially hydrolysed, the relative values for 60 minutes being 9.5 and 17 respectively. As the final degree of hydrolysis observed was practically the same however in *B* and *C* it is extremely improbable that any of the enzyme had been destroyed during the experiment and the difference in the two rates must be attributed to the influence of the products present when the addition of glycogen was made. This view is supported by the next experiment.

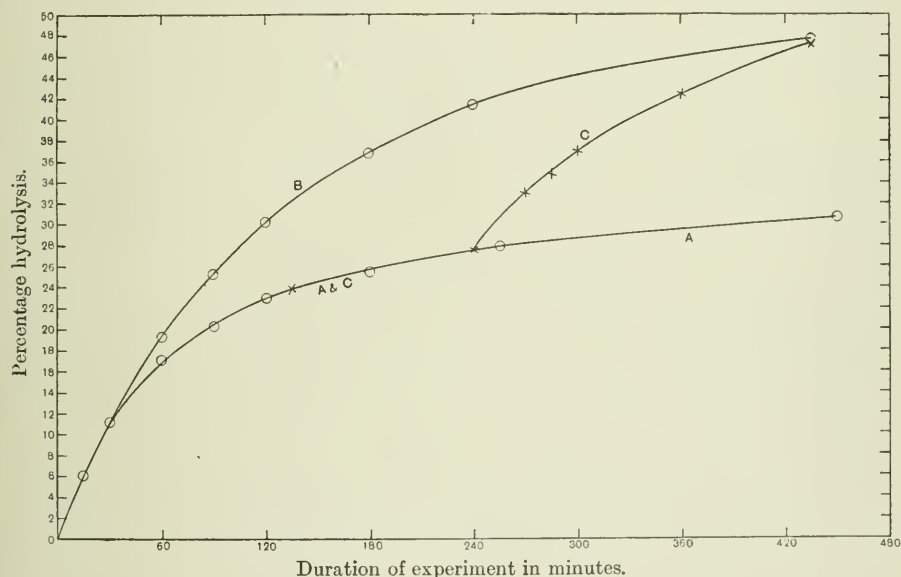


Fig. 2.

Exp. 6. Addition of glycogen and enzyme at the "stationary point."

A. 100 cc. 1 per cent. glycogen + 1 cc. enzyme + 1 cc. toluene.

B. 100 cc. 2 per cent. glycogen + 2 cc. enzyme + 1 cc. toluene.

C. Initially as *A*. When the action had slowed down, enzyme and glycogen were added to make the proportion of these similar to those obtaining in *B*.

It was to be expected that the glycogen added to *C* would be hydrolysed with a considerable initial velocity but that this rate would fall off quickly owing to the influence of the products on the freshly added enzyme. For the

same reason it was unlikely that the final degree of hydrolysis would be as great in *C* as in *B*.

An examination of the results shown in Fig. 3 indicates that these hypotheses were verified. Here again the ordinates represent the degree of hydrolysis expressed in the percentages of one gram hydrolysed to maltose.

The glycogen and enzyme were added to *C* after 300 minutes and while the glycogen was readily hydrolysed the rate was actually less than that obtaining initially in *A* which had only half the concentration of enzyme. This result can only be explained by the influence of the products as whatever

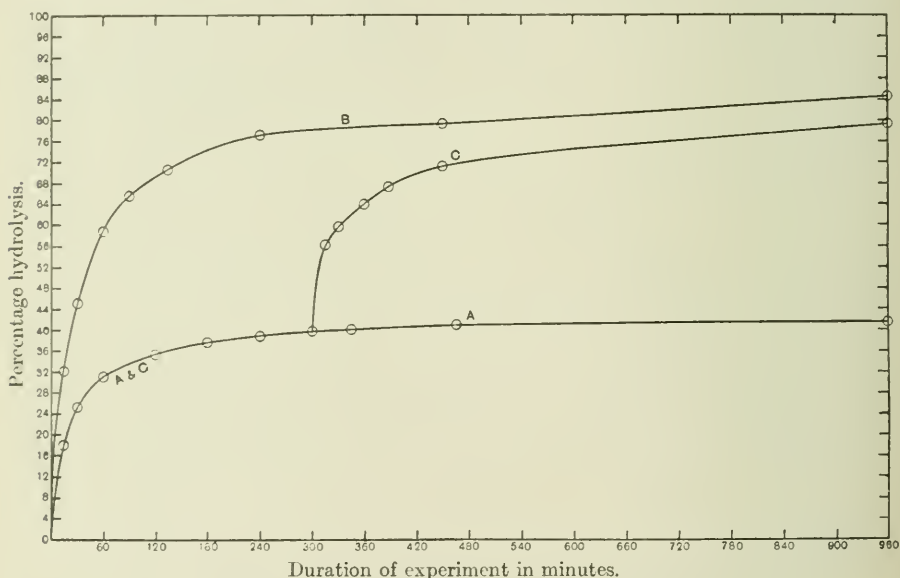


Fig. 3.

may have happened to the first quantity of enzyme during the course of the experiment, that added later was in an active condition and yet could not give such a high rate of hydrolysis as a similar quantity of enzyme acting on a solution containing none of these products. Doubtless for the same reason the final degree of hydrolysis in *C* was less than that observed in *B*.

Influence of the hydrolytic products. (a) Maltose.

Exp. 7. A. 100 cc. 1 per cent. glycogen solution + 1 cc. toluene + 0.5 cc. enzyme.

B. 100 cc. 1 per cent. glycogen solution + 1 cc. toluene + 0.5 g. maltose + 0.5 cc. enzyme.

The only difference between the two experimental mixtures is that *B* contains initially an amount of maltose corresponding to that found at the conclusion of a hydrolysis with this strength of enzyme. The rates at which hydrolysis proceeded in *A* and *B* were then compared, the results being shown in Table V.

TABLE V.

Duration of experiment	mg. Cu from 20 cc. solution	
	A.	B. Corrected for added maltose
15 mins.	27	28
30 „	46	46
1 hr.	66	65
2 hrs.	80	80
4 „	92	91
8 „	100	99
20 „	108	104
Percentage hydrolysis	49.6	47.6

This quantity of maltose had then very little depressing action on the rate of hydrolysis.

(b) *Influence of mixed products.*

Exps. 8 and 9. 200 cc. of 1 per cent. glycogen solution containing 2 cc. enzyme and toluene were incubated at 37° for 24 hours. The solution was then boiled and the degree of hydrolysis accurately determined.

The following experiments were then carried out.

A. 100 cc. 1 per cent. glycogen solution + 1 cc. toluene + 1 cc. enzyme.

B. 100 cc. boiled solution containing hydrolytic products + 1 g. glycogen + 1 cc. toluene + 1 cc. enzyme.

Two similar experiments were carried out, the results being shown in Table VI.

A control was carried out in which the boiled solution of products alone was incubated with enzyme but practically no further change took place during the course of the experiment.

In Experiment 9 there was a slightly higher initial proportion of products than in 8. In both cases however there is a well marked retarding influence exerted by the hydrolytic products, the differences observed at the end of the experiment being as follows :

Exp. 8. 5.69 in a hydrolysis of 46.55 = 12.2 per cent.

Exp. 9. 6.26 in a hydrolysis of 47.10 = 13.2 per cent.

It is possible that the freshly formed products may have an even greater influence than those examined in the experiments where the solutions had been boiled.

TABLE VI.

Duration of experiment Minutes	Experiment 8		Experiment 9	
	Percentage hydrolysis			
	A.	B.	A ₁ .	B ₁ .
15	21.40	19.0	18.0	15.2
30	29.49	26.6	27.1	21.87
60	33.77	29.55	33.28	27.65
120	38.57	33.37	38.57	32.78
175	—	—	39.03	33.35
240	40.93	34.3	—	—
360	42.78	36.2	—	—
510	43.25	37.15	—	—
675	—	—	43.72	37.9
1375	—	—	47.1	40.84
1380	46.55	40.86	—	—

SUMMARY AND CONCLUSIONS.

1. A solution of glycogen is not completely hydrolysed to maltose by the glycogenase of the pancreas unless very high concentrations of enzyme are employed, although, even with low enzyme concentrations, the whole of the glycogen may disappear.

2. The total degree of hydrolysis produced rises with increasing concentration of enzyme but not proportionally.

3. In dialysed solutions the same result is obtained by increasing the salt content up to a certain optimum concentration.

4. Evidence is brought forward to show that the resistance of the residual dextrin to further hydrolysis is not the main factor in the stopping of the reaction.

5. There is little evidence indicating that destruction of the enzyme takes place to any considerable extent during the course of the experiment.

6. The mixed products formed have a marked retarding influence on the velocity of the reaction and are probably the most important factors in the stopping of the hydrolysis. Maltose alone however has a very small effect.

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**Studies on Avian Hæmoproteozoa: No. III.—
Observations on the Development of Try-
panosoma noctuæ (of the Little Owl) in
Culex pipiens; with Remarks on the Other
Parasites occurring.¹**

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With Plates 29—31 and 1 Text-figure.

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PREFACE.

I PROPOSE in the present paper to conclude the account of the work on the parasites of the Little Owl (*Athene noctua*) which I carried out at Rovigno during the spring and early

¹ For No. II of these Studies, "On the Trypanosome of the Little Owl," etc., vide 'Quart. Journ. Micr. Sci.,' vol. 57, 1911, p. 141.

summer of 1909. I have delayed publishing up to the present my observations on the developmental phases of the Trypanosome in the mosquito, as I hoped to be able, before now, to obtain the corresponding phases, and, indeed, the transmission back again to the bird, of *Trypanosoma fringillinarum*, here in England, to complete the work. This latter research, however, progresses, unfortunately, very slowly, so that I think it useful to publish my earlier observations without waiting longer, more especially as the only other worker who has written anything of late on this subject, namely, Mayer (2), in his account of the parasites of another owl (*Syrnium aluco*), has upheld the view that the Flagellates occurring in mosquitoes which have fed on an owl are developed from *Halteridia* present in the blood of the bird. In my "Notes on Sporozoa, No. IV" (14), dealing with the nuclear structure of *Halteridium* and *Leucocytozoon*, I think I have shown clearly that, considered from the standpoint of these parasites, everything is against such a connection of either with a Trypanosome. And the evidence which I have obtained from the Trypanosome side of the question is equally negative, and does not bear out Mayer's contention in the slightest degree.

GENERAL ACCOUNT OF THE EXPERIMENTAL WORK.

The time at my disposal for experimental work with *Culex* was only very short—about the last three weeks of June. Earlier than this the bred-out females would not take blood, and at the beginning of July I was unfortunately obliged to leave Rovigno. Five owls were used, to which it will be convenient to refer by their numbers, viz., 15, 16, 19, 22, and 23. The first two were quite free from any infection; No. 22 had only *Leucocytozoon*, while Nos. 19 and 23 both had *Halteridium noctuæ*, *Leucocytozoon ziemanni*, and *Trypanosoma noctuæ*. As regards Owl 19, the Trypanosome was for some reason or other excessively scanty, and was not, I believe, present at all in the general circulation

(cf. Study No. II). The Halteridial infection of Owl 19 was quite typical, the parasites, which, of course, were in the usual form of male and female gametocytes, being fairly numerous, and many of them full-grown and ripe for liberation from the host-cells. On the other hand, the Halteridial infection of No. 23 was not typical. In this owl nearly every red blood-cell was infected, usually with several (four or more) small forms, which in many cases, as they had increased in size, had united together into a kind of common plasmodium. This condition has been already described by me elsewhere (13). I observed hardly any full-grown, normal gametocytes in the blood of this bird. Owls 22 and 23 only arrived on June 12th.

Early in June, finding that my bred-out females would not yet bite, I left Owl 19 one evening in a cage into which I had put half-a-dozen or so "wild" females, caught in the same little outhouse, used as a "dark-room," where Schaudinn himself had caught many in time past. Before midnight two of these had fed. I may note here that the favourite place for feeding of the mosquitoes was the fleshy pad just above the bird's nostrils. One of the two females was examined after about thirty-six hours had elapsed, this being the period when, according to Schaudinn, the ookinetes become transformed into Trypanosomes. Its stomach contained a number of fully-developed ookinetes, i. e. which had lost all the pigment. The majority had the characteristic curved form, but did not appear at all active. A few, however, showed a certain amount of activity, which consisted in tending to straighten out, and again recurve, the body, either slowly or now and again spasmodically. I never observed any marked forward progression of the ookinetes.

Besides the ookinetes certain other bodies were found to occur, very scantily, in the preparation. These elements were more elongated and spindle-shaped, somewhat resembling an Indian club in form, one extremity tapering finely, the other being rounded off. Further, some of them were very slightly curved or crescentic. Apart from the difference in

shape, the general appearance of these bodies, observed living, was not at all unlike that of the ookinetes. They were quite non-motile. While I was in the act of observing one, and wondering whether it was a later stage in the development of an ookinete, it gave one or two very slight, jerky movements, and before I had fully assured myself that these really represented active, voluntary motion on the part of the parasite, to my great surprise it had developed a flagellum. I thought I had just an indication of the tapering end of the body beginning to lengthen, but more than this I did not see. One moment the parasite had no flagellum, an instant later it had a fully-developed free flagellum, about three-quarters as long as its own body. The process must have been exactly comparable, in short, to that which I have since found to occur in *Leptomonas* ("Crithidia") *fasciculata* (vide 15). I hurriedly brought a colleague, Dr. Reichenow, then also working at Rovigno, to look, and he, in turn, saw the process repeated in the case of another individual. These were the only two instances in which we saw the development of the flagellum, and we only found one other flagellated form in the preparation. We both carefully examined several of the other (non-motile) elements, and satisfied ourselves that they had no sign whatever of a flagellum. If I may be permitted the personal reminiscence, I well remember how, in the excitement of the moment, we were both of us firmly persuaded that we had seen the most important stage in the transformation from an ookinete into a Trypanosome, as it had been described by Schaudinn. This confidence did not endure, however, for many days. I continued to watch certain ookinetes during the afternoon, and felt very disappointed that I could not see any indication whatever of a typical ookinete passing into one of the fusiform bodies. After keeping the preparation under observation for about three hours I removed the coverslip and made smears, which were fixed and stained.

Most unfortunately, Owl 19 was taken ill and died on the following day, and for some days I had no owl infected with

Trypanosomes. During this interval I fed several caught mosquitoes on an uninfected bird (either 15 or 16), and these were examined at periods of from thirty-six to fifty-eight hours after being fed. In two cases, elongated, fusiform elements, perfectly similar to those above-mentioned, were found; but none of them was seen to become active or develop a flagellum. In another female, examined about fifty-four hours after being fed, numerous active Flagellates were observed in the stomach; these differed considerably in appearance from the "resting Flagellates," but, on the other hand, agreed closely with the characteristic developmental forms described below, and I have not the least doubt that they also belonged to the life-cycle of some Avian Trypanosome. It is important to note that no ookinetes were seen in any of these mosquitoes. These observations showed not only that the fusiform resting Flagellates might probably have another origin than from the ookinetes of *Halteridium*, but also that it was essential to use only bred-out females, in order to follow the course of development taken by the blood-parasites in the *Culex*; hence, from this time onwards only such individuals were used.

On June 12th Owl 23 arrived, and during the night of the 15th-16th I found Trypanosomes in the peripheral circulation; the parasites were of the characteristic fusiform, rather stout type (Pl. 31, fig. A), described by Minchin and myself (l. c.). The Trypanosomes were not at all infrequent—for Avian Trypanosomes, it must be remembered—in the peripheral blood at this time,¹ and were found also on other occasions. In both of the two first mosquitoes which were examined after being fed on this bird, after intervals of about thirty-four and forty hours respectively, numerous active Flagellates were found in the stomach. Digestion was proceeding normally and was about half accomplished, or rather more. (I may state here that females which had taken blood were always kept at a tem-

¹ In any living preparation, consisting of a small drop of blood spread out into a thin layer under a coverslip seven-eighths of an inch square, there would be one or two Trypanosomes.

perature of from 25°-27° C., at which temperature digestion took three to four days to be completed.) The development of the Trypanosomes appeared to be at about the same stage in both the females, and in both similar phases were observed.

Ookinetes of *Halteridium* were also found, but in both mosquitoes they were few in number—quite scarce, in fact, when compared with the number present in the first female examined, which had fed on Owl 19 (cf. above). This was readily to be understood, bearing in mind the different condition of the Halteridial infection in the two birds; in spite of the very strong infection of Owl 23, there were nothing like so many full-grown, ripe gametocytes as in Owl 19. A living preparation made from one of these two stomachs was kept under observation for some time. Three ookinetes in different fields, all of which had lost their pigment grains, were noted at intervals during two hours, but none of them showed any change in form or the slightest indication of any development into one of the fusiform bodies or into a flagellate condition. The preparation was again looked at two hours later, with the same result in the case of two of the ookinetes; the third could not be found. By this time most of the Flagellates seemed to be dead—at all events, only two or three could be observed, and these were very sluggish.

Altogether, twenty-six female *Culex pipiens* which had fed on Owl 23 were examined, after intervals varying from about twelve to eighty hours. Flagellates were found in twelve of these, i. e. in about 46 per cent.; sometimes they were numerous, in other cases only few were seen. On the other hand, out of thirty-two females fed on one of the other owls (Nos. 15, 16, 22), none of which was infected with Trypanosomes, the stomachs of which were carefully examined after different intervals (thirty-six to fifty-four hours), in not a single case were the parasites found! Two of these birds were quite free from any Hæmoprotozoan infection; Owl 22, however, had a fairly strong infection of *Leucocytozoon ziemanni*. Seven of the thirty-two mosquitoes examined

had fed on this latter bird,¹ and in four of them a few of the large ookinetes of *Leucocytozoon* were found. In form and appearance these were very similar to the ookinetes of *Halteridium*, but they were considerably larger. Those I observed were quite motionless, and did not change at all in shape.

The fifty-eight mosquitoes examined were barely half the total number (about 120) which fed on blood. The mortality amongst these newly bred-out imagines was high, and it appeared to make little difference whether they took blood or the sugar-water, banana and prune juice with which they were supplied. More than a quarter of those which fed on blood died during the course of digestion. During the short time at my disposal I had, therefore, only very limited material for transmission-experiments. During the last fortnight between fifteen and twenty females which had fed on Owl 23 and successfully completed digestion, were given the opportunity of feeding on an uninfected bird (either 15 or 16). To my very great disappointment, however, not one of these could be induced to feed again. Some of them drank a little water, or partook of the food-supply which was placed in the same cage for a few hours during the day-time for the males to feed upon; nevertheless, many of them gradually died off during this period. I was loath to sacrifice any more for examination, hoping to the very morning of my departure from Rovigno that one or more would bite again and give me the chance of seeing whether one of the uninfected birds would become infected. Unfortunately, the endeavour did not succeed. Owls 15 and 16 accompanied me back to England and lived for many months—much longer than a single bird did at Rovigno—but neither of them ever showed any parasites at all.

While restricting myself to bred-out females for the experimental work, incidentally I examined a few more caught "wild" mosquitoes. None of them was infected with active Flagellates (i. e. Trypanosome developmental forms), but again

¹ This owl died on June 22nd, so that I had it only ten days.

two or three contained the peculiar, spindle-like resting forms, above noted; in no further instance, however, did I see one develop a flagellum. I have since wished that I had been able to devote more time to the study of this parasite, and to ascertain, for example, whether it occurred in male mosquitoes also. But I was intent on proving the origin of the Flagellates which developed in the blood-fed females, and in transmitting them back to the birds, if possible, and this took every minute of my time, as I had no assistance whatever and had everything to do myself.

DESCRIPTION OF THE PARASITES, AS THEY WERE FOUND IN THE
MOSQUITOES.

(i). *Trypanosoma noctuae*.

I pass on now to describe the developmental phases of the Avian parasites in the female *Culex pipiens*, and begin with those of *Trypanosome noctunae*. Considering the Flagellates, first of all, as they were seen in life, in mosquitoes examined about thirty-six hours after being fed on an infected owl, the most striking form, which at once held my attention on first seeing it, was a very long, extremely slender type, which progressed rapidly, by means of its flagellum and the undulating membrane along the anterior part of the body. The membrane appeared to be very narrow along the middle region of the body, and on account of this fact and the active movements of these individuals, I could not determine exactly where it ended, or whether this type was trypanomonad (erithidial), or trypaniform. In fixed and stained preparations, however, it is seen to be distinctly and invariably trypaniform (figs. 13-21). Nevertheless, these attenuated trypanosomes were entirely different in appearance from an ordinary, slender, elongated blood-form of *Trypanosome*, e. g. a piscine type such as *T. granulosum*. While the anterior part of the body, where the membrane was conspicuous, was sinuous and flexible; the hinder part, as a rule, nearly half

the entire length or more, was held quite stiffly and did not appear to be actively flexible at all. Frequently it was practically straight, but in some individuals it was quite curved round, like a crook (cf. figs. 19-21); this posterior part would retain this shape, unaltered, even while the parasite was moving rapidly forwards. From a comparison of stained preparations it is evident that this portion of the body represents a prolonged extension of the cytoplasm behind (posterior to) the kinetonucleus. In view of my observations on the living parasites, I regard this cytoplasmic "tail" as differing from the anterior half of the body in lacking anything of the nature of myonemes, and consequently any ability to bend or twist of itself. I consider that it is only, as it were, passively flexible, and that any curving or bending is produced mechanically, as the result of contact with the blood-cells and other elements among which the parasite happens to be working its way. What function this remarkable development serves, I was not able to ascertain.

The above type of individual constitutes a fair proportion of the total number of flagellates present, even at this somewhat early stage of the development. The other types of parasite seen were for the most part relatively short trypanomonad (crithidial) forms and individuals representing every possible transition between such and the extremely attenuated forms. As is shown by the fixed and stained preparations made of stomachs at about this period, the majority of these intermediate forms are really trypaniform, i. e., the kinetonucleus is on the aflagellar side of (posterior to) the trophonucleus. The shorter, more typically trypanomonad individuals resembled the commonly occurring crithidial forms which develop in cultures of an Avian trypanosome, such as I have described in the case of *T. fringillinarum*. All these forms had what is usually distinguished as the crithidial type of movement. Progression was in a slightly zig-zag manner, the flagellum and the anterior part of the body (corresponding to the position of the undulating membrane)

vibrating actively. The movement of these forms was not nearly as rapid as that of the long, slender individuals. Lastly, other forms noted were short and pyriform, and moved jerkily, not displacing themselves to any extent; these were infrequent. Notwithstanding the great increase in number of the parasites which must have taken place since the blood entered the stomach, I found scarcely any individuals actually dividing. In one or two cases I saw trypanomonad individuals with two flagella, and in one instance a pair of such forms still connected together; in another instance which I noted, division was markedly unequal, a short, pyriform individual becoming separated off from a distinctly larger, broader, club-shaped form.

At a later period of the development the long, attenuated forms predominate more and more, until in females examined fifty-five hours or subsequently after being fed, the stomach contained apparently only such forms; and this is borne out by the study of fixed and stained preparations made of stomachs of this period or later.

All my observations on the parasites relate to the stomach-phases of the life-cycle which occur between thirty-two and about seventy-six hours after the mosquito has fed. I examined four females from twelve to eighteen hours after feeding on Owl 23, but I could not find any Trypanosomes at this early period. It would be necessary to examine many individuals to find the earliest changes in the parasites, because, even if the development is proceeding all right, the Trypanosomes have not yet had time to multiply and give rise to any considerable number of parasites; and it must be remembered that only very few Trypanosomes are taken up by the mosquito from the blood to start with. Neither did I find any phases of the parasites in the intestine; but this did not surprise me, because nearly all the mosquitoes were examined, at any rate, some time before the stomach was quite empty. While digestion is still going on, the stomach is most certainly the principal, if not the only situation in which the Trypanosomes occur. If I had been able to

examine a sufficient number of females after digestion had been completed, or after they had had another meal of blood, the parasites might have been found in the intestine; I shall discuss this point later on. My observations are, I am aware, only incomplete; but they were the fullest I was able to make in the circumstances, and bearing in mind the chief objects on which I concentrated my attention during the short time at my disposal, namely, to determine whether the Trypanosome-phases in the mosquito were derived from the Halteridial parasites or not, and to bring about, if possible, the transmission of the Trypanosomes back again to the owl.

The Trypanosomes in Permanent Preparations; Comparison with the Developmental Stages found in Cultures.—I think, however, by comparing the various forms which I did obtain in the mosquito, with the development which I have found to take place in cultures, in the case of another Avian Trypanosome, *T. fringillinarum*, that a general idea can be arrived at of the main course of the natural development in the stomach of the insectan host; as already indicated, the chief gap and element of uncertainty relates to the earliest changes undergone by the parasites.

I have no hesitation in making use of the cultural development for this purpose, because practically all the different forms occur in both cases. The reason it is helpful is because, in the culture, the development continues over a much longer period owing to the fact that there is no absorption of the medium, such as occurs in the insect's stomach, and therefore the intermediate forms are met with abundantly and stages in division are frequent, the latter being of much assistance in determining the sequence of the developmental changes. In the *Culex*, on the other hand, the course of digestion is comparatively rapid and completed in three to three and a-half days, by which time the stomach is empty of blood. It is undoubtedly in relation with this fact that we find the early and intermediate stages in the development of the parasites passed through very quickly, which leads on to the production of the ultimate stages found in the stomach. This develop-

ment proceeds along two lines, the result being the formation of two extreme types. The great difference in the relative frequency of certain forms, on the one hand in the culture, and on the other in the mosquito's stomach, is entirely in accordance with the different conditions prevailing in the medium in the two cases.

The earliest developmental forms obtained are trypanomonad individuals, such as are drawn in figs. 1-3, or *b* and *c* of the scheme. These are medium-sized, fusiform parasites, with a distinct membrane and the two nuclei close together, the kinetonucleus being usually just posterior to the middle of the trophonucleus. Both nuclei are either about the middle of the body or else slightly in the hinder part. This type of individual corresponds exactly with a particular crithidial form which occurs commonly in cultures, such as is drawn in figs. *f'-j'* of the parallel series of stages from cultures, which I have reproduced¹ for ready comparison with the developmental forms in the mosquito. In the mosquito, however, even in my earliest preparations (about thirty-two hours), the number of those trypanomonad individuals is relatively very small. This is in marked contrast to what is the case in the cultures, where the trypanomonad type is by far the predominating one. In the cultures many of these forms are distinctly larger than those with which I compare the mosquito forms just described (cf. figs. *b'-d'*), and have the undulating membrane often better developed. In a small proportion of them, moreover, the kinetonucleus is just in front of the trophonucleus, instead of being alongside, a condition which I have not observed in this phase in the mosquito.

As regards the immediate origin of these trypanomonad forms in the stomach, I think it is most probable that they arise by the division of rather larger, but otherwise similar forms. As is seen very clearly from the full series of the crithidial forms figured in my memoir (l. c., Pls. 27, 29), the

¹ All these figures of cultural forms are taken from my Memoir in the 'Quart. Journ. Micr. Sci.,' vol. 55, 1910, pl. 27, 29, or 30.

division of the larger (earlier) individuals is at first by practically equal binary fission, the only inequality being that the daughter-flagellum may be shorter than the parent one (cf. figs. *e'-g'* of the scheme, Pl. 31). Unfortunately I have not found any typical trypanomonad individuals in the act of the dividing in my preparations, but, as already mentioned, I observed an instance in life in which the parasite was just completing division, and in a condition practically identical with that seen in fig. *g'*.

However, I have very little doubt that the division of these trypanomonad individuals with the nuclei about the middle of the body is almost, if not quite, finished by the time of my earliest preparations. Because there are very few forms still left in this phase of the development; most of the parasites occurring are individuals which have lost the trypanomonad condition already and become trypaniform, and are either at some stage in the development of the elongated, attenuated trypanosome-phase, or have, in fact, attained the latter. I have been able to find, in stomachs of from thirty-two to thirty-six hours, a regular series of transitional forms in this connection (cf. Pl. 29, figs. 4-10). The elongated trypaniform condition is reached by a progressive modification of the form of the above trypanomonad individuals. I think this change is most probably unaccompanied by further division, at any rate, to any extent; otherwise, I ought to have found some indication of the process, as the parasites are fairly numerous, and all degrees in the gradual change of the type of form are to be met with.

The earliest change is the passage of the kinetonucleus, and, of course, of the blepharoplast and attached flagellum, definitely behind the trophonucleus, the latter retaining its position (figs. 4 and 5). Next, the general cytoplasm in the posterior half begins to increase considerably in length; in this way the parasite becomes longer, but it does not increase much in breadth. On the contrary, as the length increases, the body becomes ultimately not only relatively, but actually narrower and more slender. The change is most probably the

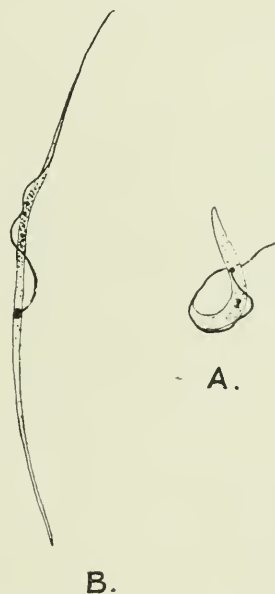
result of two factors combined, namely, growth to some extent, and a thinning-out of the general cytoplasm. The latter factor is evident from the change in the form of the trophonucleus. This body, in the early stage, has the shape of a short and broad oval (figs. 4-6); as the trypaniform condition develops it becomes elongated, and appears as a narrow oval, compressed laterally (figs. 7-10). Meanwhile the kinetonucleus has passed well back behind the trophonucleus, but it never approaches anywhere near the posterior end of the body, the extension of the cytoplasm backwards, as the "tail" develops, being so great (figs. 8-12). The final position of the kinetonucleus is usually about the middle of the body, or slightly in the posterior half. Nevertheless, the attached flagellum and the membrane are relatively long; the membrane is always narrow and rarely at all wavy. Although, as the parasite assumes its final attenuated condition, the anterior part of the body also becomes thinned out and tapering, it does not appear to increase in length to anything like the extent that the posterior half does; so that the trophonucleus lies, as a rule, distinctly in the anterior part of the body, at times comparatively near to the anterior end (cf. figs. 13-20).



As the final stage in the development of this form is nearly attained, a characteristic change usually occurs in the appearance of the trophonucleus. This organella, which has already become narrowed and compressed, becomes broken up into many oblong or rather rod-like blocks, arranged with their length transversely to the long axis of the body of the parasite, thus giving the whole nucleus a remarkable ladder-like appearance (figs. 13-17). Apparently this change represents really the break-up of the original large karyosome, which is not seen, as a rule, in the nucleus when stained by Giemsa, into several small karyosomes arranged in a row. This is shown by the comparison of wet-fixed preparations stained by iron-haematoxylin, in which slender elongated trypaniform individuals, approaching the final form, have, instead of one large karyosome (as in Text-fig. 1*a*), four or

more small karyosomatic bodies (Text-fig. 1*b*). Probably each rod or block in the Giemsa-stained parasites corresponds to a karyosomatic grain of the greatly extended trophonucleus. Whether this change in the nucleus is absolutely necessary before the final form can be said to be completely

TEXT-FIG. 1.



× 1600. Stages of *Trypanosoma noctuæ* in the mosquito, showing different conditions of the trophonucleus (from wet-fixed film, stained by iron-hæmatoxylin). A. Intermediate trypaniform phase. The karyosome of the trophonucleus is apparently budding off a small daughter karyosome. B. Approximately final (propagative) form. There are five distinct karyosomes forming the elongated trophonucleus.

formed, I do not feel certain; occasionally individuals, which in other respects seem to have reached the final stage, are found where the nucleus is not ladder-like (cf. figs. 19-21).

In my preparations of stomachs of fifty-five hours or later, scarcely any intermediate changes are present, practically all the individuals having attained the attenuated trypaniform

condition. These are certainly very striking in appearance; their thread-like form can be judged from the fact that their length is generally from 52 to 56 μ , while they are rarely more than 1 μ broad at the widest part, and often scarcely that! Superficially, they almost deserve the name of spirochætaform Trypanosomes, were that not so very misleading. The half of the body in front of the kinetonucleus—the actively motile part—is frequently spirally twisted or coiled to some extent; while the long, passive cytoplasmic “tail” behind the kinetonucleus may be either practically straight (figs. 13, 14, 18 and 20) or bent up on itself in a curve (figs. 15–17, 19 and 21).

The above-described trypaniform type also occurs in the cultural development. I have observed individuals both in the intermediate condition, and in what undoubtedly corresponds to the ultimate form attained in the mosquito's stomach (cf. figs. *k'*–*m'*). It is important to note that the proportion of such forms to the trypanomonad individuals in the cultures is just the reverse from what is the case in the mosquito after thirty-two hours. This provides a very interesting commentary on the course of the cultural development as compared with that of the natural one. Whereas, in the latter, the environmental conditions favour and lead on to the production of the attenuated trypaniform type, by the modification of the ordinary trypanomonad individuals, in the cultures there is not the same stimulus (the approaching completion of digestion) to the production of this form, and in consequence it is only developed, as it were, in isolated instances; the multiplicative (“crithidial”) form, on the other hand, persists and increases in numbers, far beyond what occurs under the natural conditions. The intermediate forms in the cultures resemble closely the corresponding individuals from the insect's stomach (cf. figs. *e*–*g*). The few final forms which I have found (fig. *m'*) also agree quite obviously in their chief features; there is the same long, cytoplasmic “tail,” and even the ladder-like nucleus may be developed. The principal difference is that the individuals in the cultures are

much broader relatively than the natural "spirochaetiform" individuals; their body has apparently more bulk. I think this is simply an indication that these forms have grown more in the cultural medium than they do in the stomach; and division having most probably ceased, the result is the marked increase in "girth."

To consider next the other line of development followed by the parasites in the mosquito, this proceeds also from the original type of trypanomonad individual, by a modification in form and the mode of division. Here, again, I think the course of events can be understood from the cultural development. In certain of the ordinary trypanomonad individuals, the nuclei show a tendency to be in the hinder part of the body. This may be in the first place due to a slight obliqueness in the direction in which the preceding nuclear division has occurred. At any rate, in such individuals the direction of the nuclear division is usually oblique, and we find not only the persistence of the nuclei in the hinder part of the body, but a distinct tendency already for the division of the body to be slightly unequal (cf. fig. *n'*). These individuals with the nuclei definitely in the posterior half of the body pass, or grow into the characteristic club-shaped forms,¹ the general body-protoplasm tending to be concentrated in the region of the nuclei (fig. *o'*). Now, in these forms, the division is always markedly unequal. It is important, I think, to note this particular point, that where the nuclei are situated about the middle of the body, the division is practically equal (as in the ordinary trypanomonad individuals); where, on the other hand, the nuclei are distinctly in the hinder part of a club-shaped individual, the division of the cytoplasm is unequal. This is seen in figs. *p'* and *q'* of my scheme and also in several other figures in my memoir (12). I consider this mode of division is primarily due to the drawn-back position of the nuclei, and consequently of the blepharoplasts, and to the fact that the new daughter-flagellum is, at any rate, largely

¹ I formerly distinguished these by the cumbersome term of "accentuated trypanomonad" individuals.

formed by free, independent growth. Individuals which are manifestly the product of a similar division are seen in figs. *r* and *t'*.

Now, what I have found in the mosquito agrees entirely with the above description. One of the earliest stages along this line of development is the characteristic club-shaped trypanomonad form of fig. 22, or *k*. This individual corresponds very closely, it will be seen, to that of fig. *o'* or *p'*; it is almost ready for division. As remarked in my account of the living observations, I saw one instance of such a form in the act of unequal division. The smaller of the daughter-individuals about to result was a short, pear-shaped form, with scarcely any membrane; the larger one was a rather club-shaped individual, long and tapering, and probably with a long, narrow membrane (cf. fig. *q'*). Unfortunately, I have not obtained any examples of individuals just dividing in my permanent preparations; in view, however, both of the living observation and of the fact that numerous individuals belonging to both the distinct forms which are produced by such division occur in my preparations (cf. figs. 24, 26, and again, figs. 23, 25), I cannot doubt that such unequal division takes place as a normal and typical phase of the development in the mosquito.

By further division of the larger daughter-individual (possessing the long membrane), smaller forms are produced (figs. 30-32 or *n* and *o*); these can at first be recognised as corresponding to one or the other of the two types, but in the smaller individuals the distinction between the daughter-forms tends to be less marked. Here and there I have found a stout pyriform individual, one of the early-developed pear-shaped daughter-forms, just about to divide, after further growth (fig. 27); and also a quite small parasite, one of the ultimate stages, I should say, undergoing division (fig. 34). There can be no doubt that this line of development leads ultimately to the production of small, pear-shaped or oval parasites, with the nuclei close together and situated about the middle of the body, or nearer the posterior end, and with

the flagellum drawn back but with practically no membrane (figs. 29, 32 and 33). Here we have, unmistakably, the haptomonad phase of the Trypanosome, as I have proposed (16) to term the so-called "gregariniiform" phase, which serves for attachment (and coincident multiplication). If these figures, for instance, are compared with certain of the text-figures in my description (15) of *Leptomonas fasciculata*, as I found this parasite in *Culex pipiens*, it will be perfectly clear that they represent the corresponding phase of the Flagellates in both cases. This agreement, I may incidentally mention, affords an excellent example in favour of the point I have urged, that from the haptomonad phase alone (having regard only to the morphology) it cannot be said with certainty whether a particular Insectan parasite represents a Leptomonad, a Crithidia or a Trypanosome.

From the above account it is clear that the early development of *Trypanosome noctuæ* in the mosquito culminates in the production of two distinct and extreme types. Whether the above description includes all the modifications of form which occur in the life-cycle in the Insectan host, I am not able to say. I think, however, that it is not difficult to interpret the significance of the end-stages, and if the view I favour is correct, any further stages in the life-history represent in the main a repetition, or re-development of the above sequence of forms, consequent on the persistence of the parasites in the mosquito and their response to fresh meals of blood.

The haptomonad forms most probably—as, indeed, is implied by thus designating them—become attached to some part of the wall of the alimentary canal, lose practically all the flagellum and enter upon the resting phase; "resting," that is to say, as regards locomotion, but not in regard to nutrition or multiplication. I think there is no reason to doubt that the chief situation favoured by these haptomonads for attachment, as the digestion becomes finished, is the anterior end of the stomach and especially the invagi-

nated epithelium of the proventriculus, as was so graphically described by Schaudinn (10) and illustrated by him in Textfig. 14. Just because the parasites were in this situation, I feel sure that in regard to this point Schaudinn's account relates to actual developmental phases of *T. noctuæ* and not to a purely Insectan form (such as, possibly, *Leptomonas fasciculata*). In the case of the latter, on the other hand, the haptomonad forms are restricted to the intestine apparently. Whether the haptomonad phase of *T. noctuæ* also invades the intestine to any extent, I am unable to say; considering that these forms tend to mass themselves at the anterior end of the stomach, it is quite likely that they do not—unless they are also able to form cysts for passage to the exterior.

Returning now to the first line of development, what is the further destiny of the remarkable thread-like individuals? I am decidedly of the opinion that they represent the propagative phase of the Trypanosome in the mosquito, the form, that is, in which the parasite is finally transmitted back again by inoculation to the owl. Unfortunately, I have no proof of this, but certain considerations point strongly to this being the right explanation of the significance of the above very characteristic type. In the first place, a brief comparison with the known course of the life-history in piscine Trypanosomes is most suggestive. It has been recognised for some time that a given species of fish Trypanosome shows—at all events, in many cases—very considerable polymorphism during that part of the cycle undergone in the blood of the Vertebrate host; thus certain individuals of a species may be quite small, slender forms (representing a young stage), others large, massive forms, with all intermediate grades between cf. *T. granulorum*, *T. percæ*, Minchin [3]. Now I have shown that the same pronounced polymorphism also obtains in Avian Trypanosomes (vide [12] and, with Minchin [5]). Whereas earlier writers frequently described a small form from a particular host as one species, and a large, massive form from the same host as another species, the true meaning

of these different types is that they are different stages in the life-history of one species. Thus the large, massive, "blue" Trypanosomes of the little owl, originally described under the name *T. ziemanni*, and connected by Schaudinn with *Leucocytozoon ziemanni*, are really only the large forms of *T. noctuæ* and not a separate species at all. And a corresponding great variation in form and size is found in *T. fringillarum*, in the chaffinch. Hence there is a strong family resemblance between piscine and avian Trypanosomes, as regards the types met with in the Vertebrate host; and it seems quite clear that both belong to the same group or division of Trypanosomes, and stand somewhat apart from Mammalian forms, for instance. Turning now to the life-cycle of piscine forms in leeches, which has been fully worked out by Miss Robertson in two or three cases (e.g. *T. raia* in *Pontobdella* [7 and 8], *T. danilewskyi* in *Hemiclepsis* [9], it is found that, after a varying period of multiplication by the parasites in the trypanomonad (crithidial) phase, as the digestion approaches completion—the time occupied varying considerably according to the conditions—a trypaniform type is developed, which becomes very elongated and slender. This passes forwards from the crop into the proboscis-sheath, and is the propagative, inoculative form, which transmits the infection to a fresh fish.

This type of form is essentially similar to that above described in *Culex*, the significance of which I believe to be also the same; in the leech it does not apparently attain the same degree of tenuity and the remarkable thread-like appearance which it does in the mosquito, but it may at times show the same peculiar ladder-like nucleus. It may at first be thought that from thirty-four hours onwards is too soon for the final, propagative form in the mosquito to be already present. But this rapid development appears quite explicable when the habits of the mosquito are considered. Unlike most of the Invertebrate hosts (transmissive agents) of Trypanosomes, mosquitoes do not feed solely on blood. On the contrary, it is generally recognised that, so far as the species of

temperate climates are concerned, meals of blood are not by any means the rule, and in many cases only taken in connection with the development of the eggs. In the case of most females blood appears to be necessary for this purpose; it certainly is so in *Culex pipiens*, and as I have recently shown (l. c.), two meals of blood will suffice to bring about the growth and oviposition of the fertilised ova. After laying one, or perhaps two, batches of eggs, most of the females either die or go into hibernation. Hence, it is perfectly clear that unless the propagative phase of the Trypanosome is developed in time for inoculation at the second (or at most the third) meal of blood, the chances of the parasite passing back to the bird are very uncertain—an entirely different state of affairs from what is the case among tsetses or leeches. For the above reasons, therefore, I think there is no difficulty in assuming that the thread-like “spirochætiiform” individuals which I have described represent the inoculative type, or in understanding their early development in the mosquito.

While I consider it is quite likely that these forms are inoculated again into the blood at the second time of feeding, I do not overlook the possibility that they (or some of them) pass first into some other organ of the mosquito (such as the salivary glands, or œsophageal diverticula), and there await a third meal; but this has still to be ascertained. I do think, however, Schaudinn was mistaken in stating that the Trypanosomes cannot be transmitted back again to the owl until the fourth meal inclusive. I should say, if the parasites had to wait until the mosquito took a fourth meal of blood, they would rarely, if ever, have the opportunity of getting back again at all, unless, perhaps, they were in a female which was going to hibernate. It is obvious from Schaudinn’s account that he was following chiefly the multiplication of the parasites in the haptomonad condition (vide his description of their attachment in vast numbers to the walls of the alimentary canal). Schaudinn does not appear to have seen the real propagative phase at all! He nowhere describes the

attenuated forms with the characteristic cytoplasmic "tail" (cf. on the other hand, Mayer's account, referred to below). Schaudinn's slender, "spirochætiform" individuals, which he describes under "*Spirochæta*" (*Trypanosoma ziemanni*), agree fairly well with the intermediate trypaniform stages in the development of the final type (such as, e. g. figs. 7 and 8); but I must add, nothing has astonished me more than the lack of close correspondence, in the main, between the different forms of the Trypanosome occurring in the mosquito, as Schaudinn figured them, and as I have found them.

II. The Ookinetes of *Halteridium noctuæ* and *Leucocytozoon ziemanni*.

A few words next concerning the ookinetes of *Halteridium* and *Leucocytozoon*, as they occur in my permanent preparations. As regards their general appearance, I have little to add to the description previously given by Mayer (l. c.). The ookinetes of both forms are fundamentally similar in type, as was of course to be expected, considering that two essentially similar parasites are concerned. In both cases the body is very frequently more or less coiled up in the form of a C. Practically the only difference between the two is that the ookinetes of *Leucocytozoon* are much larger, especially in regard to length, than are those of *Halteridium* (c. f. figs. 35-40 and 41 and 42).

In my preparations of the *Culex* which fed on Owl 19, made from thirty-three to thirty-six hours after feeding, the ookinetes of *Halteridium* are very numerous. In preparations made from mosquitoes which fed on Owl 23, ookinetes are also usually to be found (up to thirty-six hours), but they are very scanty; this difference is due to the different conditions of the infection in the two owls respectively (see above, pp. 400-401). Speaking generally, all the ookinetes observed are in the same phase of development, and with one or two exceptions have lost all the pigment. In none of them is anything like

a kinetonucleus, let alone a developing flagellum, recognisable. In a certain number a small chromatinic area, or clump of chromatinic grains, is present, in addition to the nucleus (figs. 35 and 36); but these stain quite similarly to the nucleus, and in no case have the characteristic appearance of a kinetonucleus, as seen when stained by Giemsa (contrast the "resting flagellates" referred to below). Unfortunately I am unable to say whether the nucleus possesses a central karyosome or not, but at any rate there is certainly no excentric or extranuclear karyosome, such as occurs at certain periods both in *Halteridium* and *Leucocytozoon* when in the blood,¹ and which often simulates a kinetonucleus in so marked a manner that it was formerly mistaken for one, until I showed clearly (14) what its true significance was. Frequently the ookinete shows one or two vacuoles in the cytoplasm; I have not been able to find any ookinetes in my preparations of mosquitoes which were made later than fifty-four hours after feeding.

I have only come across very few ookinetes of *Leucocytozoon* on my smears; I was generally able to find one or two during the living examination of the stomachs of mosquitoes which had fed on an owl infected with this parasite, but unfortunately in several cases, owing to their scantiness, I have not succeeded in obtaining any on the permanent slides made. Those I have found are all practically similar in form and size (figs. 41 and 42). The nucleus is always situated fairly near to the more rounded end of the body, as it is also in *Halteridium*; it varies in size to some

¹ Reichenow, in a note on *Leucocytozoon ziemanni* in his account of the Hæmogregarines in 'Handb. d. Path. Protozoen' (6) states that he was unable to find a karyosome in the male gametocytes. As I showed in my "Notes on Sporozoa," published about the same time, there is certainly such an organella present, excentric or even extranuclear as in the female forms. It is unmistakable in preparations stained by iron hæmatoxylin, but it is rarely shown in Giemsa smears. Reichenow does not say whether the figure he gives is from a Giemsa smear or not, but from its general appearance (e. g. the hypertrophied host-cell nucleus stains quite differently after iron-hæmatoxylin), I should say it was.

extent. Now and again separate chromatinic granules occur close to it (cf. fig. 42). In these ookinetes, also, one or two vacuoles are often present. None of the ookinetes of *Leucocytozoon*, any more than those of *Halteridium*, are in any later stage of development. I have not seen the least indication of the remarkable skein-like formation so graphically described by Schaudinn, accompanied by nuclear multiplication and the eventual development of a number of very small Trypanosomes! I must say that I doubt very much now the correctness of all this.

III. The "Resting Flagellates."

As was described in the account of the living observations (see above, pp. 401-402), in a small number of female *Culex pipiens* caught wild, which were examined at an early stage of the experimental work before I restricted myself to the use of bred-out mosquitoes, certain characteristic and rather peculiar motionless bodies were sometimes present. I have mentioned how on one occasion such resting forms were actually seen to develop into active flagellates, and the temporary illusion fostered by the observation. But not only were they seen in the first "wild female," which fed on Owl 19, well infected with *Halteridia*, they also occurred now and again in females which had fed on an uninfected bird, or which were examined before being allowed to feed on a bird at all! Hence, whatever their origin, there is no reason for associating them with *Halteridium*. For some cause or other, with the above-noted exception, these resting forms were never observed to become active, nor were active flagellates corresponding to them ever found.

In my permanent preparations, practically all these parasites occur in the resting condition (figs. 43-45): in one or two instances, however, there is an unmistakable wavy border in the anterior part of the body, accompanied by a drawn-out, tapering anterior end (figs. 46-47). I am strongly of the opinion that the flagellum is actually developed in both

these cases, but it is difficult to be quite certain because for some reason or other, it has not stained red in the usual manner after Giemsa. Especially in the individual drawn in fig. 47, however, a definite line is evident along one edge of the anterior, tapering part, which is continued free for a short distance, which in all probability represents a flagellum. The general appearance of these forms, moreover, closely resembles that of the particular individual referred to above, just the instant before a well-marked, free flagellum became apparent. I think no one can have any doubt that these are really Flagellates, because they are certainly binucleate forms; in all cases, a definite kinetonucleus is present, usually immediately in front of the trophonucleus, which has the characteristic staining reaction to Giemsa. It may be pointed out that there is no question of this element being a karyosome, because, the karyosome of the nucleus can at times be seen, appearing, as is always the case in Giemsa-stained smears, as a clearer area, with a distinct centriole in the middle (cf. figs. 43 and 46). The cytoplasm always stains more darkly and much more bluish-purple in tint than any of the developmental phases of *Trypanosoma noctuæ*, in which, by the way, aflagellate phases appear to be entirely lacking. In all my experience of crithidial forms, whether as developmental phases of Trypanosomes, or "Crithidial" parasites, I have never observed any in which the cytoplasm stains in this peculiar dark manner, or in which the flagellum is so faint.

This parasite is certainly a crithidial form; this is evident from the contiguity of the two nuclei about the middle of the body, as well as from the distinct undulating membrane, where an individual is in, or about to assume, the active condition. In one respect it appears to be unique, i. e., in being non-flagellate, and moreover, without any trace of a rhizoplast, and quite motionless, when still possessing the elongated, fusiform shape, which is always associated in other Flagellates of this kind, with the active, flagellated condition; in all other cases of which I am aware, when the

parasites are "resting" and non-flagellate they are in the short, more or less oval, haptomonad phase. This form does not agree with any of the "Crithidiæ" hitherto described from mosquitoes; until more is known about it I am inclined to regard it as a distinct parasite. At present I have obtained the impression that this may be a purely Insectan form, a parasite more particularly, perhaps, of the larval *Culex*. Somehow, it does not look like a form accustomed to a blood-medium; its appearance and behaviour are so different from all the other crithidial forms which I have had occasion to study.

GENERAL CONCLUSION REGARDING THE QUESTION OF A CONNECTION BETWEEN THE TRYPANOSOME AND THE HÆMOSPORIDIAN PARASITES OF THE LITTLE OWL.

For the last time, I hope, that it will be necessary, I return to this subject, more particularly in order to point out the bearing upon it of the observations recorded above on the developmental phases of these parasites in the mosquito. It must be apparent, indeed, that these observations support and further strengthen the conclusion at which I had already arrived (14), that there is no connection whatever between these different types of parasite; and I have found nothing that in any way corroborates Mayer's account (2), in which he has upheld the opposite view.

In the first place, with regard to the mosquito which fed on Owl 19. As was to be expected from the typical ripe Halteridial infection of this bird, numerous fully-formed ookinetes were found without difficulty in the stomach when dissected. But not one of these ever showed any sign of passing into a flagellate condition; nor were any active Flagellates observed of the different types which I subsequently found frequently in mosquitoes fed on an owl known to have Trypanosomes. This fact is very significant when it is remembered that no Trypanosomes were ever seen in the blood of Owl 19, either in life or in searching smears, and if

they were present, must have been so rare at the time as to be negligible.

As was discussed above, the matter was complicated just at the outset by the use of a few "wild" females in which a "resting Flagellate" occurred, which on one or two occasions developed into the active condition. In my own opinion, however, it is perfectly clear that these resting Flagellates have nothing to do with the ookinetes. Although there is undoubtedly a general resemblance in appearance between these two bodies when observed in life, there are several important reasons for concluding that this is only a coincidence. In stained preparations the two types of element appear fundamentally distinct, for the resting Flagellates show without exception the binucleate condition; the ookinetes, on the other hand, never do. In not a single instance, whether of *Halteridium* or of *Leucocytozoon*, have I been able to find an ookinete which possesses the binucleate condition, the first essential for it to be regarded as connected with a *Hæmoflagellate*. Again, the staining reaction of the general cytoplasm in the two cases is entirely different; and though, knowing what I do of the dangers, no less than the advantages, associated with the use of the Giemsa stain, I should be the last to lay stress upon casual staining differences in different cases; nevertheless, where such a difference is constant and uniform, weight may be laid upon it. Further, these resting Flagellates occurred also in "wild" females fed on owls totally uninfected with any of the parasites under discussion, and in which no ookinetes, of course, were found. Finally, these resting Flagellates have certainly nothing whatever to do with the developmental phases of *Trypanosoma noctuæ*.

Now, in the bred-out mosquitoes which fed on Owl 23, ookinetes were always scanty; particular individuals were carefully watched on different occasions, but in these also no change or development of any kind was ever seen. Nevertheless, in about 46 per cent. of these mosquitoes examined, active Flagellates were observed in different stages of development; and it is to be re-

membered that Owl 23 was the only bird in which Trypanosomes were found in the peripheral circulation, and actually at this period. If the Trypanosomes had indeed developed from ookinetes, it is difficult to understand why they should occur frequently when the ookinetes were scanty (and sometimes not actually noticed), and yet not at all in the case where the ookinetes were numerous (the female which fed on Owl 19, with the normal Halteridial infection). All my observations point clearly to these active Flagellates being the developmental forms of *T. noctuæ* in the mosquito, and derived directly from the stumpy, or stout fusiform Trypanosome, present in the blood in the summer (as previously described (5)). I have never once seen the slightest indication of these developmental Trypanosome-stages in any mosquito fed on a bird which did not contain this stumpy, transmissive phase of the Trypanosome in the peripheral circulation—whether it contained Halteridium, or Leucocytocoon, or neither.

A few remarks, in concluding this discussion, about Mayer's account (l.c.). The most important statement of this worker is that, in hanging drops of blood from an owl infected with Halteridium, in which careful examination failed to reveal any Trypanosomes after four days or so Flagellates were found to be present; the inference is, of course, that these had developed from the Halteridium-ookinetes. I can only say that I feel absolutely convinced that Mayer was mistaken in supposing no Trypanosome-individuals to be present in the drop at the beginning of the experiment. I know well from experience how easily one of these forms can be overlooked, especially if the blood-corpuscles are in a fairly thick layer. Another possible explanation would be that certain very minute or ultramicroscopic phases of the Trypanosome were present, which later gave rise to flagellates. But this idea is quite unnecessary when I have shown clearly that the same particular flagellate developmental stages occur regularly, both in cultures and in mosquitoes, as a result of inoculation with a definite Avian Trypanosome form. For my own part,

I say candidly that I can see no sufficient evidence up to the present for believing in "cryptotrypanosomiasis"¹ or in "infective granules," etc. In all the life-cycles of Trypanosomes which are now known in the invertebrate host, there is no suggestion of such a thing, and such an explanation is not, I consider, required. In birds, and even more so in cattle and sheep, Trypanosome individuals may be so excessively scanty in the circulation that the greatest difficulty is entailed in finding them; but let a single individual succeed in passing into a culture or into its right invertebrate host, and it will multiply so rapidly that before many days have passed the forms to which it gives rise are readily found. I may say also that I am no longer inclined to think that any small form of these Avian Trypanosomes occurs in the red blood-corpuscles, which might perhaps be mistaken for a young stage of Halteridium. Since very considerable doubt has been thrown upon the occurrence of such a stage in Trypanosoma cruzi, it is becoming more and more probable that Trypanosomes do not get into the red cells at all; at all events I am disinclined now to postulate the occurrence of such a phase in the Avian Trypanosomes which I have studied until we have an authentic instance of it in some other case. As in the case of fish-Trypanosomes, with which, as I have shown above, Avian Trypanosomes have much in common, I firmly believe that unless the transmissive trypanosome-form of an Avian Trypanosome passes into culture or the invertebrate host, no development of Flagellates will occur.

Mayer describes and figures further certain "large forms" which developed in mosquitoes, which he regards as the developmental stages of Leucocytozoon, in contradistinction to Halteridium. But these forms are obviously the same as certain of those which I have described and figured as the developmental stages of Trypanosoma noctuæ; his fig. 56, for instance, represents an individual nearly arrived at the final attenuated form. The stages which Mayer associates

¹ By this term I understand some definite phase of the parasite, hitherto unrecognised and possibly ultramicroscopic.

with *Halteridium*, equally with those which he associates with *Leucocytozoon*, constitute part of a regular series, and belong to a definite life-cycle, as I have clearly shown. Correspondingly, it will be remembered, Minchin and I showed also that the small forms of the *Trypanosome* in the blood, associated by Schaudinn with *Halteridium noctuæ*, form part of a regular series with the large individuals, regarded by him as belonging to *Leucocytozoon ziemanni*, and altogether represent only one species—*Trypanosoma noctuæ*; there is no species *T. ziemanni*. If, therefore, one still held to the idea that the small *Trypanosomes* are connected with *Halteridium* and the larger ones with *Leucocytozoon*, one would be led to the impossible position that *Halteridium* and *Leucocytozoon* are different phases of one and the same thing.

I do not suppose that Mayer any longer considers that an ontogenetic connection exists between any of these different parasites, especially as in addition to the above-mentioned paper, I have also published my account of the cytology, in which I have shown that, after all, there is no nuclear dimorphism in *Halteridium* and *Leucocytozoon*, and that these are not related to the *Binucleata*. But as Mayer's account is the only one published of late years on the developmental stages of these parasites of the owl in mosquitoes, I have been obliged to point out where it is erroneous in the light of my own observations on the whole subject.

The conclusion of the whole matter is, that the three parasites of the little owl—*Trypanosoma noctuæ*, *Halteridium noctuæ* and *Leucocytozoon ziemanni*—are entirely distinct and separate types; and the same is undoubtedly true for other species of these parasites in other birds. So far as *T. noctuæ* is concerned I have been able to outline above the main course of its development in the mosquito (*Culex pipiens*), though there are, unfortunately, gaps still to be filled up. The development of the *Halteridium* and the *Leucocytozoon* in the mosquito remains to be ascertained—supposing, that is to say, that

there is any development beyond the ookinete stage. Other workers (e. g. the Sergeants (11), Aragao (1) and Mayer (loc. cit.) have obtained the development of one or both of the parasites up to the same stage, but never any farther. As regards *Halteridium*, Aragao, who has succeeded in transmitting *H. columbæ* from a Hippoboscoid fly (*Lynchia*) back again to the pigeon, is doubtful whether there is really any further development in the Insectan host; it is possible, moreover, that the so-called schizogony in the lung represents the delayed sporogony of the ookinetes, as has been hinted at by Minchin, in his text-book on the Protozoa (4). If that be so, then I see no reason why the ookinetes of *H. noctuæ* should not behave similarly; in which case, *Culex pipiens* may prove, after all, to be a true host—transmissive agent—of this parasite also. At any rate, for all that one can yet say to the contrary, the ookinetes may be inoculated back again into an owl, at the same time as the final propagative forms of the Trypanosome. It is, perhaps, not without significance that in every owl which was infected with the one parasite, we found the other also to be present (cf. Minchin and Woodcock, loc. cit.). The development and transmission of *Leucocytozoon* are still more a matter of uncertainty. As far as Schaudinn's observations are concerned, if such a remarkable nuclear multiplication and skein-development does occur, it is very strange that neither Mayer nor I myself have seen any signs of it. One thing is, I think, practically certain; if the ookinete does produce a number of small elements by rapid division, these will not prove to be Trypanosomes or other binucleate Flagellates!

THE LISTER INSTITUTE;

May 12th, 1914.

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EXPLANATION OF PLATES 29–31.

Illustrating Dr. H. M. Woodcock’s paper on “Studies on Avian Hæmoprotezoa : No. III.—Observations on the Development of *Trypanosoma noctuæ* (of the Little Owl) in *Culex pipiens*; with Remarks on the Other Parasites occurring.”

[All the figures on Pls. 29 and 30 are magnified 2000 times linear; those of the scheme, Pl. 31, are $\times 2000$ (nearly). I am indebted to Miss Rhodes for kindly drawing and colouring most of the figures of the *Halteridium ookinetes*.]

PLATE 29.

All the figures relate to the development of *T. noctuæ* in the mosquito (*Culex pipiens*).

Figs. 1–3.—Typical trypanomonad forms.

Figs. 4–6.—Earliest stages in the development of the trypaniform type.

Figs. 7–10.—Intermediate trypaniform stages.

Figs. 11, 12.—Approximation to the final inoculative form. Fig. 11 shows an intermediate stage in the change in the nuclear condition; three fairly large chromatinic masses (karyosomes) are present.

Figs. 13–17.—Typical attenuated thread-like forms, with ladder-like nucleus. These are considered to be the inoculative type.

Figs. 18–21.—Individuals which agree in character with the final forms, except for the fact that the nucleus has not become ladder-like.

Figs. 22, 23.—Club-shaped trypanomonad forms. Early stages in the second line of development. (See text.)

Figs. 24, 26.—Pear-shaped forms, with the kinetonucleus tending to be in front of the trophonucleus and with hardly any membrane. These individuals result from the smaller member of an unequal division of

such a form as that of fig. 22. They represent the beginning of the haptomonad phase.

Fig. 25.—Individual resulting from the larger half of the same or a similar unequal division. It tends to be slightly club-shaped, and has the nuclei well back, with a long, narrow membrane.

Fig. 27.—Pyriform individual commencing division.

Figs. 28, 29.—Other pyriform haptomonad forms.

Figs. 30, 31.—Small individuals which still show to a slight extent the characters of the differing products of an unequal division (probably of such a form as that of fig. 25).

Figs. 32, 33.—More haptomonad forms, or more strictly, perhaps, forms which are going to become attached.

Fig. 34.—One of the smallest forms found undergoing division.

PLATE 30.

Figs. 35–40.—Ookinetes of *Halteridium noctuæ*. In fig. 38 part of the skin or pellicle of the ruptured erythrocyte is seen still attached to the ookinete. This occurs now and again.

Figs. 41, 42.—Ookinetes of *Leucocytozoon ziemanni*.

Figs. 43–47.—“Resting Flagellates” found in “wild” mosquitoes. The individuals of figs. 46 and 47 have almost certainly developed a flagellum, marginal, if not free.

PLATE 31.

Scheme comparing the development of *T. noctuæ* in the mosquito with the developmental stages of *T. fringillinarum* as found in cultures. (For explanation, see text.)



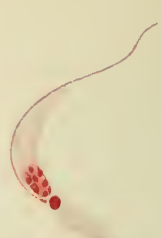
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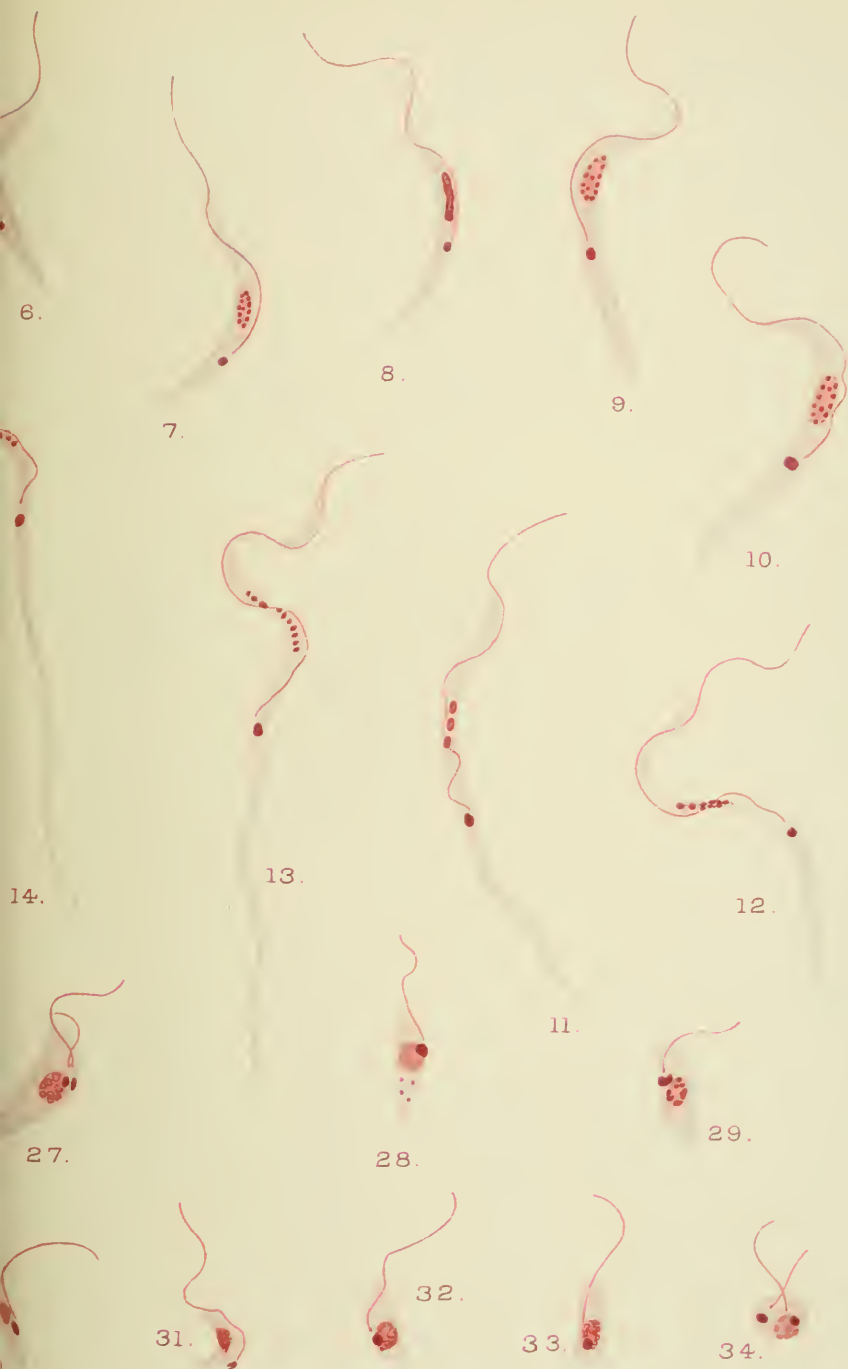
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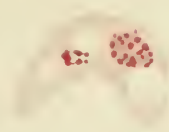


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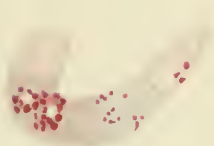
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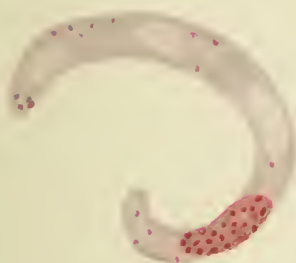
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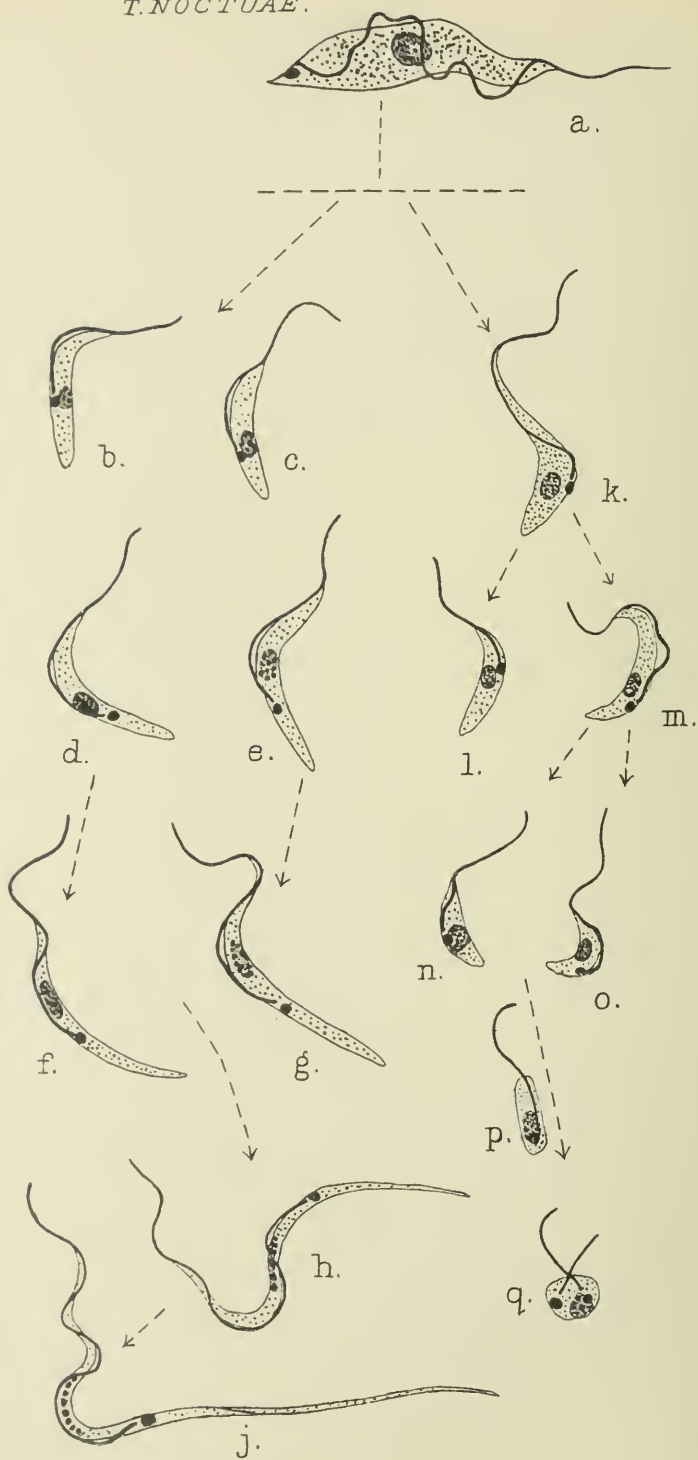


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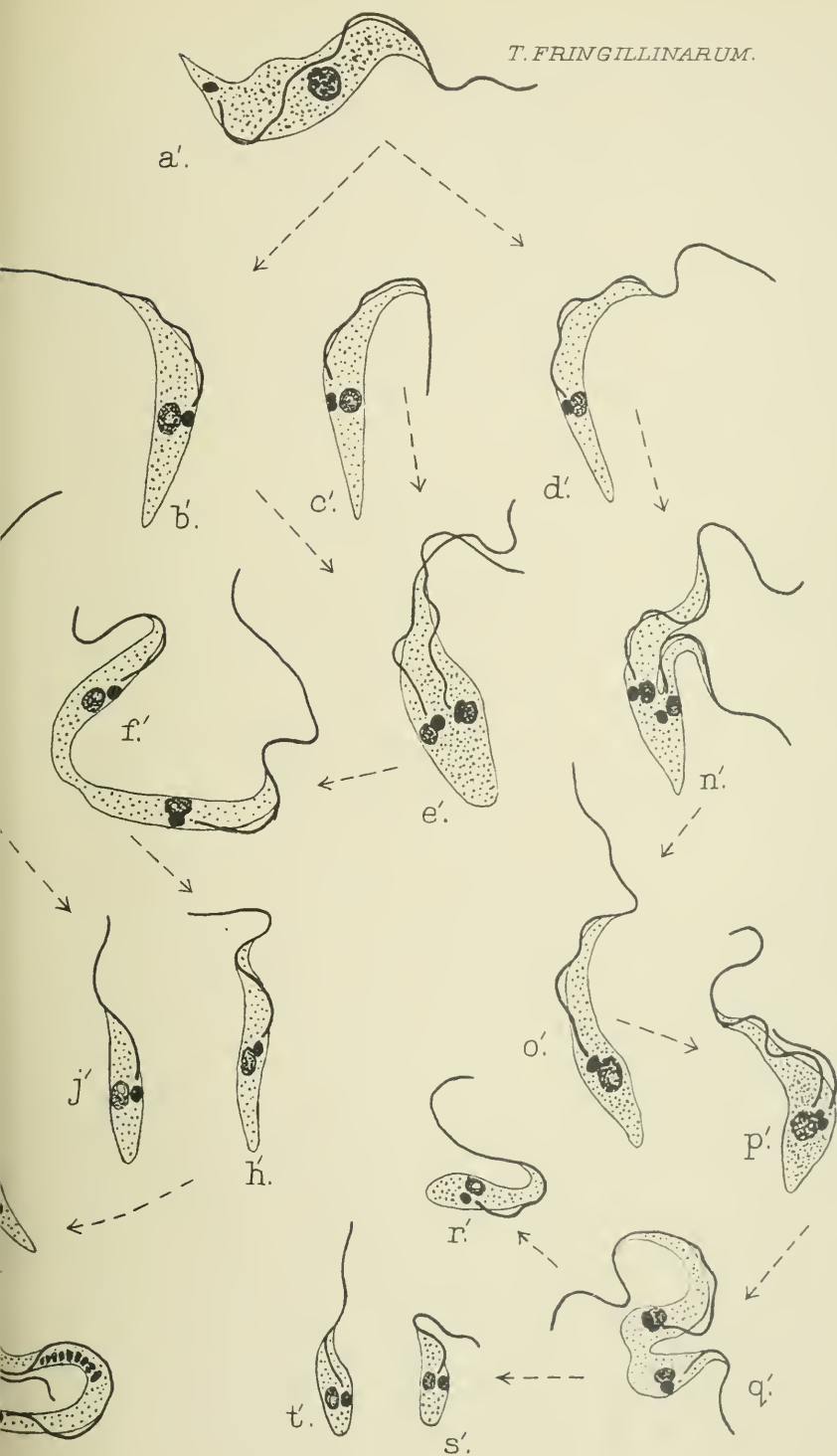
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T. NOCTUAE.

DEVELOPMENT IN THE MOSQUITO.



T. FRINGILLINARUM.



DEVELOPMENTAL STAGES IN CULTURES.

Observations on the Life-Cycle of a New Flagellate, Helkesimastix
faënicola, n.g., n.sp. : Together with Remarks on the Question
of Syngamy in the Trypanosomes.*

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(Communicated by Prof. S. J. Hickson, F.R.S. Received July 31, 1913.)

[PLATES 13 AND 14.]

This new flagellate occurs frequently in goat-dung; we have found it also in sheep-dung. It is a "passenger," being carried passively through the alimentary tract, in an encysted condition. When the dung is moistened with water—probably, in nature, when it is deposited on damp grass or earth—the flagellate emerges from its cyst and goes through its life-cycle, ultimately encysting again. The cysts are doubtless swallowed by the goat with its fodder. We have cultivated *Helkesimastix* under various conditions, which will be described in our full account later. In order to obtain this form in large numbers and study its life-history without any fear of being misled by stages in the life-cycle of other flagellates, we succeeded in isolating it from the other forms occurring in simple dung-cultures and cultivating it on agar-media, on which it multiplies rapidly. The medium which we have used principally is weak Lemco-agar, *i.e.* the same medium as used for blood-agar, but considerably diluted. For our continuous observations we have used hanging-drop preparations in sealed cells; in these cases we used very dilute Lemco-broth, without any agar, as the medium for the development of the flagellates, because in the denser agar-medium it is very difficult to see the flagellum. In all these media the flagellate forms the protozoan component of a "mixed culture," since there is, of course, an even greater and more rapid bacterial development.

Commencing the account of the life-cycle with the permanent cyst, this is a small spherical or slightly ovoid body about $3-3\frac{1}{2}\mu$ in diameter (figs. 1-3). The cyst-wall is well defined, but not very thick; it appears to consist of a single membrane, there being no differentiation into inner and outer envelope such as is found, for example, in many *Amœba* cysts. Sometimes bacteria are adherent to the cyst-wall (fig. 3). The protoplasm is

* The generic name is formed on the analogy of the Homeric epithet, *ἐλκεσιπτελος*, trailing mantle or cloak. We are indebted to Prof. Minchin for suggesting this appropriate name.

fairly homogeneous, or contains fine granules. There is frequently, however, a conspicuous, somewhat refringent grain, situated near the periphery (Plate 13, figs. 1 and 2), but no vacuole is present. The nucleus can usually be seen as a clearer area, and at times the contained karyosome can be distinctly made out as a dull body in the centre. No division takes place inside the cyst, which is therefore a resting or "Dauer" cyst and not a multiplicative one.

Excystation.—We have followed the process of excystation in cysts which had been for about 22 hours in fresh medium (Liebig broth). This is not quite the minimum period required, before the flagellate will emerge from its cyst, for in this particular preparation some half-dozen individuals were already active by this time, though the great majority were still encysted. The period which must elapse depends, we consider, upon how soon the multiplication of the active bacilli in the new environment has taken place to a sufficient extent to produce, in sufficient quantity, the ferment or chemical substance in solution which is either directly or indirectly the cause of the dissolution of the cyst-wall. For we are strongly of opinion that the explanation of both the encystment and the excystation of *Helkesimastix* (and probably equally of other dung- and infusion-flagellates) is to be found in the view put forward by Cropper and Drew* in their recent important and suggestive work on the causative factors of the corresponding phenomena in *Amoeba*. We hope to study our new flagellate from this biological standpoint subsequently, and will here merely indicate in passing certain facts we have observed which bear upon the question. We have found excystation occurring only in those cultures, plates, or observation-preparations in which there was a plentiful development of an (or of more than one) active, markedly aerobic bacillus, which we have not yet more closely determined. We have kept cysts in ordinary cover-slip preparations in different media (the preparations being sealed, of course, to prevent evaporation), for several days, without the emergence of any active individuals taking place; and in such preparations, the medium not being in contact with free air, no noticeable development of active bacilli took place.

In a cyst from which the flagellate is about to be liberated the wall is noticed to become gradually less and less obvious, until at length one can no longer say that there is any envelope present, apart from the delicate membrane or pellicle limiting the body-protoplasm (fig. 4). There is no definite rupture or bursting of the wall at any point, through which the flagellate could pass out to the exterior; in other words, when the creature has again become active and moved off, there is no cyst-wall left behind. We were the

* 'Researches on Induced Cell-reproduction in *Amoebæ*,' London, John Murray, 1914.

more convinced of this because we had previously seen the excystation of a species of *Bodo* from other dung-cultures, and in that case the rupture of the cyst-wall was very evident, and when the *Bodo* had moved away the empty cyst-wall was left behind. To return to *Helkesimastix*, when a sphere from which the cyst-wall had disappeared was watched it was seen gradually to change its shape, and in about seven minutes had elongated somewhat and become ovoid (fig. 5). Near one extremity a small vacuole had made its appearance, which represented the contractile vacuole: active metabolism had again begun. Very slight, spasmodic movements were noticed at intervals, and then suddenly it was seen that a very short, delicate flagellum was present, apparently emerging from about the middle of the body (fig. 6). This waved slowly to and fro, though we do not feel at all certain that it was causing the jerky movements. The flagellum could at first be made out only when it was projecting from the side of the creature, and not when it lay over the body-protoplasm. Meanwhile the flagellate was growing in size rapidly, and five or six minutes later it appeared as in fig. 7: the flagellum had lengthened considerably and was more prominent and stouter, and its point of origin was now near to the anterior end. The nucleus had doubtless also passed towards the anterior end, but unfortunately it could not be clearly made out: this was owing partly to the jerky movements and partly to the fact that the protoplasm was becoming very granular. The jerky, to and fro movements of the body, producing no displacement of the creature, are very characteristic of certain phases; we describe them as "knicking" movements (*cf.* below, p. 358). Ten minutes later the flagellate was beginning to make short, gliding movements of progression, and these alternated for some time with the knicking movements until, after about an hour, it moved steadily for the first time out of the field of vision.

During this period the same process had been going on throughout the preparation, and many actively gliding individuals were now present. These were all growing rapidly before beginning to multiply, and attained a size considerably larger than the average individual size found later on; the protoplasm was usually full of refringent granules.

Form and Structure.—The body of the ordinary active individual of *Helkesimastix faecicola* is typically elongated and fairly cylindrical, with one end (the anterior one) bluntly rounded, the other (posterior) one tapering away more and being at times somewhat pointed (figs. 8, 10, 13). The broadest part of the body is generally nearer to the anterior end. We can most aptly compare the form with that of the fleshy part of a small carrot. Sometimes the hinder extremity is drawn out into a narrow prolongation (fig. 11). This hinder part of the body is often very plastic and irregular,

and reminds us somewhat of the posterior extremity of *Cercobodo* (*Cercomonas*), with its long cytoplasmic "tail," though in *Helkesimastix* it is never, in normal conditions, drawn out to anything like the same extent. The body is usually about 6μ to 7μ long by $2\frac{1}{2}\mu$ or 2μ broad. Under other conditions the shape of the body may be oval or slightly pyriform (figs. 9, 18); this is frequently the case in smaller individuals, and also when the flagellates are sluggish, in a rather denser medium than usual.

There is a single flagellum, generally about two and a half to three times as long as the body, or even longer, inserted at, or very near to, the anterior end. This new flagellate is remarkable in having its flagellum *always* directed backwards, *i.e.* it possesses only a trailing flagellum, whence the generic name. In life, the flagellum is usually contiguous to the body for practically the entire length of the latter, and the proximal portion is always in very close contact with about the anterior third or so of the body, from which it never becomes free. This is seen very clearly when a steadily progressing individual makes a sharp turn and goes off in another direction. The proximal portion of the flagellum also turns immediately, along with the body, but the remaining part is for the moment free from the latter and turns more gradually into the new direction (*cf.* fig. 14). The flagellum lies along the middle of the upper (dorsal) side of the creature; in ordinary conditions it is never on the under side. Sometimes the flagellate turns on its side, when the close adherence of the flagellum is well shown (fig. 10). Yet, in spite of this close contact, there is certainly no attaching membrane developed, for, in individuals killed by osmic acid, the flagellum is sometimes seen to stand off completely from the body (*cf.* fig. 12). Frequently there is a definite row of three or four conspicuous granules along the line of contact of the body with the flagellum (figs. 10, 11). The nucleus can generally be seen as a clear, spherical area near the anterior end; in individuals in motion it is sometimes difficult to see the karyosome, but in those quiescent, or in individuals killed by osmic acid vapour, this body can also be made out (*cf.* fig. 12). *Helkesimastix* is not a binucleate; it has no kinetonucleus.*

There is certainly no cytostome or definite mouth-aperture present. We are not quite certain, however, whether the creature does or does not ingest solid particles, such as small cocci, etc.; we are strongly inclined to think it does not. In this connection, a peculiar mode of food-ingestion which we have observed in *Cercomonas* (or *Cercobodo*) *longicauda* is of interest. While

* We may add that, as seen in stained preparations, the nucleus is of the usual flagellate character, consisting of a nuclear membrane, a clear zone (the nuclear sap), and a large central karyosome, connected with the membrane by delicate radiating fibrils.

an individual was moving sluggishly along, the posterior, plastic part of the body would come into contact at some point with a small extraneous particle. A small portion of the protoplasm of the flagellate in the immediate neighbourhood of the point of contact would remain adherent to the still stationary particle for a few seconds, gradually engulfing it, while the *Cercomonas* moved calmly on. Thus most of the body was quickly separated from the small portion of cytoplasm remaining behind, until often only an extremely thin thread or line of protoplasmic substance still joined the two parts; this thread might be as long as the whole body of the flagellate. Suddenly the tension of the connecting thread overcame the resistance of the stationary particle, and the latter, together with the small portion of protoplasm surrounding it, was safely hauled up again into the main body. We often thought the thread must break, but it never did!

As our new flagellate shows a resemblance in some respects to *Cercomonas*, we have watched particularly for the occurrence of anything corresponding, which, however, we have never seen, although analogous appearances are seen during conjugation (*cf.* below). We have never been able to satisfy ourselves that *Helkesimastix* does engulf solid particles at the hinder, plastic end, but do not deny the possibility of it doing so. We certainly consider, however, that its principal mode of nutrition is by osmosis. The natural habitat of the creature, namely, moist dung, is, of course, rich in organic matter in solution, when the bacteria have been active for a little time. In *Helkesimastix*, therefore, we have an instance of a form which is, at all events, mainly saprozoic.

The contractile vacuole is usually small, that is to say, it contracts before it attains a large size. It is generally situated in the hinder part of the body, to one side (figs. 8, 10). Now and again, however, owing doubtless to some condition of the medium, the contractile vacuole becomes very large (fig. 15). Immediately after it has ruptured the protoplasmic wall and its contents have passed out to the exterior, the body of the flagellate presents for a short time the curious appearance of fig. 16; the hinder end is forked, like the two arms of a V. After a little while it becomes triangular (fig. 17), and ultimately assumes again its normal shape.

Another point illustrating the looseness or plasticity of the body is a method of turning round often shown by a sluggishly moving individual. It will come to rest, and the body becomes more ovoid (fig. 18). Then the side with which the flagellum is in close contact begins to show what we term "working" movements; at first the peripheral protoplasm moves in slow, short, irregular waves to and fro, the line both of the contiguous part of the flagellum and of the row of granules (if these are present) becoming meanwhile indented and

uneven (fig. 19). These peripheral movements of the protoplasm can perhaps be compared, on a very small scale, with the wave-like movements of the ectoplasm of *Amœba verrucosa*. Next, the anterior portion of the body-protoplasm on this side is moved as a whole backwards, around the central part, as it were, carrying with it the anterior part of the flagellum and the granules, and also, doubtless, the nucleus (*cf.* fig. 20). Finally, the creature elongates again, having the anterior part of its body now where the posterior part formerly was (fig. 21), and is ready to swim away in just the opposite direction.

Movements.—*Helkesimastix* possesses two distinct and characteristic methods of locomotion. One, the more usual mode, is very interesting, because it is very difficult to explain; in fact, we do not know quite how to explain it. The creature glides forwards with a steady, almost unwavering movement, the flagellum trailing behind in a straight line. While the rate of progression is not as rapid as that of a *Monas* or a *Bodo*, for example, the movement cannot by any means be called slow; indeed, it is often surprisingly fast, considering how little there seems to be to account for it. There is certainly no vibration of the free, distal part of the flagellum at all; in this method of movement, the flagellum does not act as a pulsellum. The creature is always at the surface of the medium when moving in this way (or it is gliding along the under surface of the cover-slip), the flagellum in both cases being uppermost. This fact leads us to think that surface-tension plays some part in this type of movement. The body is, as it were, suspended along its length to the flagellar thread, as a gymnast may be suspended along a tight rope. The body is often seen to swing sideways partially around its flagellum, appearing then as in fig. 10; but it never swings right above the flagellum. The only movement of the body which can be noted is a very slight "knicking" movement of the anterior end, *i.e.* the anterior end may make little tentative jerks from side to side, perhaps caused by slight contractions of the anterior end of the flagellum. But the anterior end of the body is not displaced laterally by more than half its width, if as much, and the strength of these slight movements appears wholly insufficient to produce the steady forward progression; moreover, the creature may glide for quite a considerable distance without even these.

The other characteristic mode of progression occurs when an individual is in the middle of the fluid, *i.e.* completely surrounded by it. Then it performs vigorous, more or less undulatory movements of its whole body and flagellum, the latter, as regards its applied portion, never becoming separated from the body, and with its free, distal part lashing actively behind. The movement of the body is very like that of a fish's tail, and not at all unlike the

movements of certain trypanosomes. Yet, in spite of all this activity, the rate of progression is no greater than is obtained by the quiet surface gliding.

One other variety of movement—not of progression—remains to be noted. An individual which has been gliding about will become anchored by the end of its flagellum to some particle of débris or small clump of bacteria. The body will then execute sharp bending or knicking movements about its narrow, posterior end, where the flagellum becomes free, often turning through nearly 180°, and then turning back again. This anchoring process recalls the anchoring of some *Bodos* by the trailing flagellum, but the body movement in *Helkesimastix* is not at all of the vibrating or dancing character seen in the *Bodo*, because it lacks the anterior, vibratile flagellum.

Multiplication.—After the flagellates have been active for some hours multiplication begins. We have not observed it occurring up to six hours after excystation has taken place, but by the end of 20 hours it was proceeding actively and had evidently been going on for some time. An individual about to undergo division always comes to rest in the first place. The body becomes ovoid and then practically spherical (figs. 22, 24, 27). The subsequent course of events is not always quite uniform, though the variations are only slight. In the great majority of cases, by the time the body-form has become rounded, or even after it has been ovoid for a short while, the flagellum is no longer visible. The free part has entirely disappeared, and we are strongly inclined to think that the attached part also goes, though we cannot write with absolute certainty because it is extremely difficult to detect a motionless flagellum lying over, and closely applied to, the body. However, we think that, in many cases, it is probably entirely withdrawn and absorbed, especially as, just prior to this stage, small “working” movements of the peripheral protoplasm occur at the side where the flagellum lay. At this stage the clear nuclear area can frequently be seen to be elongated and to possess now two karyosomatic bodies (fig. 22), the parent-karyosome having already divided. Next, the body begins to elongate again (figs. 23, 29), and in a minute or two more becomes slightly dumb-bell shaped (fig. 25). The two daughter-nuclei are practically reconstituted and have begun to separate by this time (figs. 23, 29); they can usually be seen because the body remains motionless during this period. (We hope to give information with regard to the cytological details both of multiplication and conjugation in a subsequent memoir, when we have studied fixed and stained preparations). The time of appearance of the first new (daughter) flagellum varies somewhat. As a

rule, it does not appear until the dumb-bell stage is reached, when it can suddenly be seen projecting out from the side of the body, a short distance from one end (fig. 23), and waving slightly to and fro. Occasionally, however, it can be seen while the creature is still rounded (fig. 24), and in such a case it may represent the old flagellum, which has been only partially withdrawn. The constriction at the middle of the elongated body now rapidly increases (figs. 25, 30), and about this time the second flagellum appears, always some distance away from the first and in the other half of the body, not far from the second nucleus. Very generally, the second new flagellum projects out from the side of the body opposite that where the first one is. The flagella increase in length and the body undergoes little irregular, jerky movements. Its middle part becomes narrower and more drawn out, the whole body having now the appearance of a double pear. Usually the two flagella have their free ends directed towards the middle, the bluntly rounded extremities becoming the anterior ends of the two daughter-individuals; though sometimes the second daughter-flagellum (the later developed one) starts from near the middle of the body, *i.e.* near to the constriction connecting the two halves, and points outwards. Ultimately, helped by the movements of the flagella, the two halves of the body are drawn still farther apart and the small daughter-individuals at length separate, gliding away in opposite directions. The cytoplasmic "tail," which each at first possesses, rapidly contracts and the typical body-form is attained (fig. 31).

In the above type of division, which is the most usual one, we regard the cytoplasmic fission as being approximately transverse to the original long axis, so that in this case we have the flagellate undergoing transverse division of the body. The whole process is fairly rapid. From the time when an individual has become ovoid and practically motionless to the time of separation of the two daughter-individuals only 10 to 15 minutes usually elapse. Now and again, however, the process is slower, taking upwards of 25 minutes; but this is of rare occurrence. In such a case, moreover, we have noticed that the body becomes divided before the second flagellum is formed, so that one daughter-individual swims away, leaving the other motionless for a few minutes longer, until it has developed its flagellum.

On one or two occasions we have observed a modification of the above method of division, a second flagellum being formed while the old one is still present, having only been withdrawn (shortened) a little (fig. 32). The shortening proceeds further (fig. 33), but in this case the old flagellum is not entirely absorbed but forms the basis of one of the daughter-flagella. In this variety of division we regard the fission of the body as being more in the long

axis, rather than transverse. This may represent a more primitive mode, which has been largely relinquished in favour of the other.

Syngamy.—In a fresh culture (either agar-plate or observation-preparation), after the flagellates have once emerged from their cysts, multiplication goes on, often at first with amazing rapidity, for two days or so, until by about the third day—or sometimes even earlier—an epidemic of conjugation sets in. The only important point in the whole life-cycle in regard to which we are not yet certain is whether definite conjugating individuals, gametes, morphologically distinctive from the usual forms, are developed, and, if they are, whether these are anisogamous or not. Our difficulty arises from the fact that in a culture in which conjugation is beginning, the flagellates present show more or less the customary variation in size and form; and, further, we have not yet succeeded in seeing two individuals actually come together and unite. In most of the cultures in which we have observed conjugation, the majority of the individuals belong to one of two slightly different types. One of these is rather characteristic and distinctive, we think, of this period. It has the posterior end of the body gradually tapering and always turned definitely to one side (figs. 34, 35), usually, though not invariably, the right side, when the flagellum is dorsal or uppermost. The curved tail-portion differs from the irregular extension sometimes seen at the hinder end in ordinary individuals (*cf.* fig. 11), in being fairly constant and not so changeable or metabolic, now retracted and now drawn out, as in that case. The other type is distinctly smaller, but is not so readily distinguishable from an ordinary individual (fig. 36); it is oval in shape, and the hinder end is usually more bluntly rounded.

In an observation-preparation in which syngamy has begun, two individuals are often noticed to come into contact and glide along together for a short while (fig. 37). The members of such a pair are very frequently—though, again, not invariably—of the two distinct forms just noted. The larger one of the two often appears to stick, or become attached to the other by its curved, hinder end (fig. 38); when this happens, both individuals get very excited and actively jerk themselves about for a moment or two; then they will either separate abruptly or glide along together for a short distance again, and then move apart. Unfortunately, we have never seen this process followed by actual union, and therefore cannot say whether it represents a tentative seeking out of each other by definite gametes. Only in one case, up to the present, have we caught the two gametes in close contact, with the body-protoplasm of each still separate just for an instant before joining up (fig. 39); and, in this case, so far as could be gathered from our momentary impression before the two protoplasmic masses ran together, as it were, into

one, the two conjugating individuals were not very dissimilar. We leave the matter there for the present, but hope to settle it before publishing our detailed account.*

Of course, the actual coming together and uniting is a matter of a few seconds, and therefore difficult to catch: but the gradual fusion of the two gametes and the subsequent development of the zygote is a long process, and we have observed every stage in it, on many occasions. The actual cytoplasmic union is lateral (fig. 40), and immediately after it has occurred one would think that a single protoplasmic entity was now constituted. But the subsequent behaviour is amazing and unique, so far as we are aware, in the history of conjugating elements, and well illustrates the looseness of the first union and the fluidity of the protoplasm in *Helkesimastix*—and, we doubt not, in other of these dung- and infusion-flagellates. As soon as the actual cytoplasmic union has occurred, the definite form of the two gametes is practically lost, and there is for some time a remarkable lack of attraction, or reluctance to unite, between the two essential parts, if we may thus regard the nuclei and associated elements together with the portion of cytoplasm immediately surrounding them. (This is probably because the nuclei have not yet undergone a process of maturation.) We cannot do better than describe the sequence of form-changes undergone by the zygote immediately following the union, in the instance referred to.

The conjugants of fig. 40 had not been joined for more than a minute or so before they separated again in the anterior region, one individual being desirous of steering off to the right, while the other preferred to keep straight on (fig. 42). This little difference being composed and the two individuals joined up again, one began to lag behind the other, the combined body appearing now like an irregular rhomboid (fig. 43). The conjugants were progressing forwards, more or less steadily, all the time. In the next instant the smaller half had slipped still further back, and the body had now the appearance of fig. 44. A few seconds later it was quite behind the larger half (or individual), the two being connected together only by a narrow cytoplasmic thread (fig. 45). After progressing thus for a short distance the smaller half rapidly overtook the one in front by a kind of "slithering" movement along it, and the protoplasm of both again joined up along the side (fig. 46). A few seconds later a very characteristic stage was reached, in which the combined body of the two conjugants

* We have since reconsidered the above point and now think that the tendency to adhere in couples in this way is probably purely a matter of surface tension or attraction. We have observed the same phenomenon in observation-preparations of a non-conjugating strain of a closely allied species (*vide p. 366, et seq.*).

was almost square, the whole zygote, with its two flagella trailing along near the outer sides, having almost the appearance of a procession banner (fig. 47). (At a later stage the body becomes more definitely rectangular and banner-like.) In this particular instance the zygote remained thus for a couple of minutes or so, moving along steadily, the two "halves" with their flagella at times approximating slightly, causing the common cytoplasm to sag, as it were, just as a banner does when its pole bearers do not keep their line. Now and again the zygote turned on its side, when it appeared as in fig. 41. At length it altered again completely, a portion of the body, apparently about a third of the bulk, advancing quickly in front of the remainder (fig. 48), until the two portions were only connected by a very narrow thread of cytoplasm, which was at first extended along and in contact with the greater part of the flagellum (fig. 49). We have no doubt that in this remarkable dissociation of the zygote into two portions, one nucleus goes with each half, just as the origin and proximal part of the flagellum can clearly be seen to do. Unfortunately, we could not make out the nuclei at all during these living observations of the conjugation-processes; this was due partly to the fact that in the earlier stages the conjugating pairs are very active and constantly undergoing form-changes, and partly because, in the later stages, the nuclei are most probably undergoing maturation prior to nuclear fusion. This separation, which may amount almost to disruption, of the common cytoplasm into two portions reminds us of the behaviour of the protoplasm of *Cercomonas* (*Cercobodo*) above alluded to, but in *Helkesimastix* it is not a passive leaving behind of a certain amount of protoplasm, but a separation actively brought about by a difference in behaviour of the two gametic energids. Here, again, in more than one instance (*cf.* fig. 54), we felt sure that actual rupture was going to occur, but we have observed this remarkable process on several occasions and the two halves never broke loose. To return to our particular zygote, the small leading portion suddenly turned right round, its flagellum becoming at the same time mostly free from the cytoplasmic thread (fig. 50), and then joined up again to the main portion (figs. 51 and 52). The zygote next assumed the form of a pear (fig. 53), and after another minute or less the stage of fig. 45 was repeated, with the difference that the smaller half was this time in front. The hinder part then "slithered" up along the other and the banner form was again arrived at.

All the above changes took place in a period of about 14 minutes from the instant of first union. This time the banner was fairly permanent, and after following it for a little while longer we left it, as we knew exactly what the subsequent behaviour would be from numerous earlier observations. The above described remarkable behaviour of the two conjugants—or, at least, of

the two essential portions, since the cytoplasm is apparently indiscriminately divided at times—is not at all an exceptional occurrence; indeed, we think something similar usually happens during the earlier stages of syngamy. On other occasions we have seen both the “slithering” of two slightly unequal portions and the almost complete separation of the two halves which remained connected only by a delicate thread, just as in the above case. The joining up again in such a case occurs, we consider, as a result of the rapid contraction of the connecting thread, in just the same manner as the lagging portion of protoplasm was suddenly hauled up into the main body of the *Cerrobodo* (*cf.* above). Further examples of these early conjugation stages are given in figs. 54–57, from different zygotes. Even after the banner has been definitely formed for as long as an hour, we have seen it suddenly break down into two portions, one behind the other.

Different forms assumed by the banner, when at length permanent, are seen in figs. 58–62. As time goes on the banner gradually loses its square shape and becomes oblong (fig. 63), the two parallel flagella also gradually coming to lie nearer to the mid-dorsal line, and so to each other. Most probably, by this time, the maturation processes of the gamete-nuclei are completed. Further, the two flagella are slowly shortening in length. In its earlier period, however, the banner is still capable of performing the active, undulating (free) movement, as well as the more characteristic gliding movement. From an oblong the banner now turns to an oval—really, of course, to an ovoid—which is ultimately almost as deep as it is broad. We have followed a banner from the time when it had about just become permanent (*e.g.* as in figs. 61 and 62), up to the definite oval (fig. 65), the time occupied being about three hours. In an observation-preparation in which syngamy has been going on for some time, banners and ovals are quite numerous; this will be apparent when it is remembered that every cyst formed is a zygote-cyst. At a later stage the two flagella have become much shorter (figs. 66 and 67), and the flagellate moves very sluggishly; it soon ceases to displace itself and only performs little turning and “knicking” movements. The protoplasm becomes contracted and somewhat denser, and the oval nearly always leaves the under surface of the cover-slip by this time and sinks to the lower level of the medium. The flagella are at length quite absorbed and the body becomes spherical. We have followed an individual oval with two flagella close together, as in fig. 65, up to the time when it became a motionless, rounded body at the lower level, and this change took about six hours. The whole process up to this stage takes from 9 to 10 hours, according to our actual observations; of course, this may not be the minimum period required, though we should say it is not far from it.

We have not actually watched a motionless, rounded zygote through its encystment period, but we noted the position of the above individual and of others at the same time late in the evening, and on looking at them next morning we found they had become the very characteristic "shrinkage" cysts (figs. 68 and 69). The cyst-wall has been formed and the protoplasm has continued to contract, so that a space is left at one side between the body and the wall. We are inclined to think it is a space containing no liquid, because it is always very clear. The space appears of different size, according to the age of the cyst (figs. 68 and 69). When small the space is spherical, but when it attains its maximum size it is lens-shaped. A curious fact is that after the cyst has shown this condition of shrinkage for some time—the period may vary from two days or less up to longer—the space disappears entirely and the cyst becomes a permanent cyst, as described at the commencement of this account. This finishes the life-cycle.

Biological Notes.—As, in a normal culture, the great majority of the flagellates form cysts, one could not have a more convincing and readily obtained demonstration of the regular occurrence of syngamy in the life-cycle of these simple dung-flagellates. Although all the above observations on *Helkesimastix* have been described either from plate-cultures or from observation-preparations, this is only because, on account of the more rapid multiplication and development under those conditions, the individuals are very much more abundant than in a simple, moistened dung-culture, and therefore the different stages are more readily found. We have not the slightest doubt that the life-cycle is exactly similar under the more natural conditions, the only difference being that it is probably slower, *i.e.* a longer period may elapse before the whole life-cycle is completed. For instance, it is usually three or four days before this flagellate is observed in the active condition in a dilute dung-culture, and it may persist in the active condition for a week or more before forming cysts. The reason for this is, we consider, because the watery medium is not nearly so rich in nourishment as the beef-extract medium. The bacterial development is not nearly so great as in the latter case, nor is the multiplication of the flagellates so abundant. Therefore, on the one hand, a somewhat longer time most probably elapses before the cyst-wall is dissolved; and, on the other hand, the medium does not so soon become excessively full of the "toxic" products, or whatever chemical substances induce the cessation of multiplication and the tendency to conjugation, whether formed from the flagellates, or the bacteria, or from both. As, however, we are at present engaged in making a full study of this interesting flagellate from a biological standpoint we will not further discuss these questions at present.

Loss of Syngamy and the Power to form Cysts.—We have discovered one very interesting and important fact, however, which deserves to be mentioned. By sub-culturing the flagellates* while they are still all in the active condition, *i.e.* before cysts have been developed, on to a fresh plate of the same medium, we have found that multiplication will continue for a further period (of two days or so), before conjugation sets in. The important fact, however, is that, after a certain number of sub-cultures have been thus made, the flagellates, although they are still able to thrive and multiply actively on each successive transference, no longer undergo syngamy, followed by cyst-formation, and have, so far as can be seen, entirely lost the power to do so, at all events under the existing conditions.† Up to the present time (October), we have thus kept a “strain” for more than 20 weeks, through 35 sub-cultures on to fresh “constant” medium, and in each sub-culture the flagellates have multiplied enormously. In the sub-cultures up to the fourth a very few isolated cysts were still found. *But in none of the subsequent ones has a single cyst ever been seen.* The flagellates, instead of conjugating and forming great numbers of cysts, as usual, degenerate and die off, only a small proportion remaining still alive. Yet, at the end of 10 or 12 days, sometimes more, a few individuals still persist alive, and the transference to a fresh sub-culture can be successfully made. The flagellates which remain alive are rounded, granular, very sluggish forms, with the flagellum sticking straight out and almost motionless; but, in the fresh medium, they are capable of quick recuperation and renewed multiplication.

During the earlier weeks, after this new strain was fairly started, we never observed any biflagellate forms, comparable to the conjugating pairs above described, although thousands of individuals must have passed under our eyes. Then, during the 8th and 9th weeks, respectively, we observed on two occasions, in different observation preparations, a single biflagellate individual. After this, we looked carefully for others, at intervals, but no more were seen until the 14th week (in the 23rd sub-culture), when another was observed. Since that time, in most of the sub-cultures, a few biflagellate individuals (banner-like forms) have been found and the number of these has gradually tended to become less infrequent, although they still constitute a very small

* This experimental work, it may be mentioned, has been carried out upon another species of *Helkesimastix*, with which we have worked latterly. This form (*H. major*, n. sp.) also occurs both in sheep and goats, and differs only from *H. faecicola* in the larger size of the adult individuals and also of the cysts, rendering it more convenient for study. The life-cycle is similar in both.

† We tried the experiment of re-introducing the non-conjugating “strain” into the original dung-culture (sterilised) with the object of seeing whether the ability to undergo syngamy would be restored. This also gave negative results.

proportion only of the total number of flagellates present. We have been able, on several occasions recently, to follow the further behaviour of these forms, and have ascertained the important fact that they always divide, and never become rounded-off and form cysts. Moreover, we have observed a certain number of these forms which were very large and possessed not two, but three, or even four or five flagella (and nuclei, as ascertained from permanent preparations). We have seen an individual with three flagella, and also one with five, actually divide into two rather unequal portions; in the former case, one half had two flagella and the other one, and in the latter case, one had three and the other two flagella (and nuclei).

We will assume, for the present, that these forms represent a union of two or more individuals, rather than a long-delayed division.* When we first observed the isolated instances of biflagellate forms in this strain, we considered that they represented true syngamy, which, although occurring very rarely was apparently still "latent." We now know (1) that these "unions" may be either binary or multiple, apparently more or less indifferently; (2) that these forms always divide, the nuclei and flagella being apportioned out, often unequally, between the two halves, and that they never proceed to form cysts—the invariable sequel, normally, to conjugation in *Helkesimastix*; (3) and, lastly, that these forms, or the products of their division, are equally incapable of persisting ultimately (unless, of course, they are transferred to fresh medium). Hence, we feel certain that none of these unions represent true syngamy. Bearing in mind the extremely plastic character of the cytoplasm of this creature, we consider these unions are due to physical, rather than "vital" factors, and result from continued cultivation and the condition of the culture at the time.

In our opinion, these peculiar cases do not invalidate the following general conclusion which we wish to draw from our observations on this non-conjugating strain. The "intensive" culture of this flagellate has resulted in the loss of the power to undergo true syngamy and to form cysts. The further existence of this "strain" is now dependent on continued transference to fresh "constant" medium. Minchin, in his 'Introduction to the Protozoa' (London, Arnold, 1912), in the chapter on "Syngamy and Sex," has pointed out (p. 161) that "intensive culture, whether artificial or

* We cannot yet write with certainty upon this point, because we think, from the evidence obtained so far, it is possible that these forms represent a long-drawn-out mode of division, due to the effect of the cultural conditions, in which the nucleus and flagellum divide, it may be more than once, before the cytoplasm does. There are other cases known of a corresponding behaviour under conditions which are probably not quite normal; an example which is particularly interesting in relation to the present discussion is that of multiple longitudinal fission in certain lethal trypanosomes.

natural, as in parasitism, seems to diminish the necessity for syngamy"; from this stage it is only a step further to find the capacity for syngamy lost.

Bearing on the Case of the Trypanosomes.—It appears to us that this remarkable experimental fact has an important bearing on the question of the trypanosomes, and may afford an explanation of why conjugation (or syngamy) in these parasites, though assiduously sought, has not been observed in any authenticated instance. Probably it never does occur, because these forms have lost the power to conjugate. Just as, in the case of the above-discussed strain of *Helkesimastix*, the rapid, successive transferences to fresh, non-toxic medium at first removed the necessity for conjugation (and encystment), and then have led to the loss of the ability to undergo syngamy (and, in this case, to form cysts), so a similar development has very likely occurred in the trypanosomes and related forms. As is obvious, the conditions of life in these parasites are readily comparable to those above described. The trypanosomes live in a rich nutritive medium, namely blood, in the vertebrate, or blood in various stages of digestion, in the invertebrate host; and, as is well known, rapid multiplication in both hosts is usually found. In the invertebrate we get, more or less frequently, replenishment, *i.e.* a fresh supply of the "constant" medium (namely blood), together with removal of toxic products. In the blood of the vertebrate, where there is at first abundance of medium, but not any "fresh" supply, it is very interesting to note that, in the case at all events of many lethal trypanosomes, which, as Minchin has pointed out, are not yet completely adapted to their hosts, we get the production eventually—after active multiplication has gone on for some time—of the well known "involution" forms. These, at any rate in our opinion, as in that of many French workers, simply die off after a time, if left to themselves; but they are capable of being "rejuvenated" and of again multiplying actively if passed into a fresh host. And a similar state of affairs has been met with by one of us in cultures of avian trypanosomes. Now it seems to us that these two cases present a very close parallel to what we have observed in *Helkesimastix*. It may be pointed out, also, that the non-conjugating strain of *Helkesimastix* is the result of an abruptly originating and more or less artificial intensive culture, whereas in the case of trypanosomes and allied parasitic flagellates the corresponding conditions have operated naturally and over a long duration of time.

On the supposition that syngamy has now been entirely lost in the hæmoflagellates, the explanation which we here put forward is much more probable, we think, than the idea, which has also been suggested, that the stimulus afforded by the change of hosts is accountable for the loss of this process in the trypanosomes. We have always considered that there are two or

three serious objections to this latter view. Briefly stated, these are as follows:—(1) Trypanosomes can be successfully inoculated, without limit, into fresh vertebrate hosts, *i.e.* the same “constant” physiological medium, without showing (so far as is known) conjugation. (2) Binucleate flagellates of insects alone (*e.g.* certain leptomonads) have no alternation of hosts, and syngamy is apparently just as little likely to be found in these parasites as in the trypanosomes themselves. (We are inclined to think, indeed, that syngamy may have been lost in the ancestral trypanosome form, before ever it acquired an alternation of hosts.) Lastly, we have the important case of the Hæmosporidia, intracellular parasites which all have the change of hosts, and in all of which conjugation regularly occurs. Why should not the physiological stimulus afforded by the change of environment have influenced them also? And, on the other hand, there is no reason to doubt that the primitive ancestors of the trypanosomes underwent a process of syngamy, since it appears likely that many of these lowly proto-monadine forms, among which the origin of the trypanosomes is to be sought, possess this feature.

We consider, therefore, developing the ideas expressed by Cropper and Drew (*loc. cit.*) along the line indicated by the experimental facts adduced above, that the loss of syngamy is due to the surfeit of nutrition, together with the non-toxicity of the medium, that is to say, the absence (in excess) of the chemical substance or substances to which the flagellates react normally by the cessation of multiplication and the onset of conjugation. It will be readily apparent, from what has been just pointed out, how these factors have prevailed in the case of the trypanosomes. And we think that a similar explanation can be applied, not only to the case of insectan Binucleata, but probably to that of many other parasitic flagellates as well.

EXPLANATION OF FIGURES.

All the drawings, with the exception of fig. 12, are from sketches made directly at the time of the living observation. Fig. 12 is a camera lucida drawing of an individual fixed with osmic acid vapour. The magnification throughout is approximately 2250 times linear, arrived at after a comparison of other individuals fixed with osmic. We are indebted to Miss Rhodes for kindly tinting and sharpening up the drawings.

In our figures, we have shown the nucleus or omitted it, according as to whether we were able to observe its position in the particular individual represented, or not. As mentioned in the text, we were unable to make out satisfactorily the nuclei in life, in the conjugating pairs.

Note with regard to the flagellum:—As mentioned in the text, the length of the flagellum varies in individuals otherwise similar. In order to save space, we have drawn the flagellum usually short, except in fig. 10; the flagellum of the individual of fig. 12 is, of course, natural length.

PLATE 13.

Figs. 1-3.—Cysts.

Figs. 4-7.—Excystation and the development of the flagellum.

Figs. 8-11.—Different forms of individuals.

Fig. 12.—Individual fixed with osmic acid vapour, to show that the flagellum is not actually attached by any membrane to the body.

Figs. 13 and 14.—To illustrate the behaviour of the flagellum in turning of the body.

Figs. 15-17.—Rupture of a large contractile vacuole and form-changes of the body.

Figs. 18-21.—To illustrate a mode of turning of a stationary individual (see text).

Figs. 22-26, 27-31, and 32 and 33.—Different instances of division (binary fission).

PLATE 14.

Figs. 34-36.—Different forms predominating in a culture about to start conjugation.

Figs. 37-62.—The whole process of conjugation (syngamy), showing the different form-changes during the progress of the fusion.

Figs. 63-67.—The gradual contraction of the body and absorption of the two flagella of the zygote, prior to encystment.

Figs. 68 and 69.—The cysts as first formed, so-called "shrinkage" cysts, with a vacuole or space of varying size.





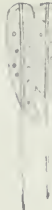
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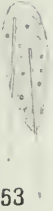
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LXV. THE RATE OF INACTIVATION BY HEAT OF PEROXIDASE IN MILK. I.

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(Received Nov. 16th, 1914.)

The presence of peroxidase in fresh cow's milk can be detected by the usual reagents for that enzyme. Hydrogen peroxide and *p*-phenylenediamine are considered the most sensitive reagents for the purpose and are mostly employed in testing milk for peroxidase (Storch's reaction). A dark blue-grey colour is imparted almost instantaneously to the milk if the reaction is positive.

That peroxidase is an actual constituent of the milk, and not due to bacterial contamination, is evident from the fact that initially sterile cow's milk, or milk rendered sterile directly after milking by antiseptics like chloroform or toluene, gives a positive reaction. On the other hand, bacterial cultures from the most common organisms in milk fail to react for peroxidase [Lane-Claypon, 1913, p. 11].

Everything seems to point to the fact that the enzyme is associated with the albumin fraction of the milk. Julius Meyer [1910] showed that when caseinogen is brought down by means of trichloroacetic acid, acetic acid, natural acidity or calcium chloride, and properly washed, it does not react for peroxidase, while casein brought down by means of rennet gives but a faint reaction. Again, when milk is centrifuged the enzyme remains in the fluid and does not go up with the cream [Lane-Claypon, 1913, p. 12]. Yet when the precipitate brought down from whey by saturating it with ammonium sulphate is redissolved, the solution reacts very intensely for peroxidase [Lane-Claypon, 1913, p. 10].

Like most enzymes, peroxidase can be inactivated by heat, or at least, when the milk is heated beyond certain temperatures for some time no perceptible reaction is manifested when the usual reagents are employed.

This fact Storch found could be utilised as a reliable test for detecting whether the milk had been heated to 80° C.

Van Eck [1911], investigating the process of inactivation of peroxidase in milk by heat, found that it proceeds as a reaction of the first order and, making use of the formula for a unimolecular reaction, was able to obtain a constant. This was confirmed by the author, who further investigated the matter with the view of ascertaining whether the peroxidase reaction in milk could be employed with any certainty as a test for pasteurisation. The temperature coefficient for the inactivation of peroxidase in milk and the influence of acidity and alkalinity and neutral salts on the rate of inactivation were studied.

EXPERIMENTAL METHOD.

The technique employed was very similar to that used by van Eck. There is no method for the determination of the absolute quantity of peroxidase, and therefore the amount of the enzyme present in the samples was determined relative to that of the raw milk used in the experiment, and expressed as a percentage of the same. It was carried out colorimetrically. For the standard scale 38 test tubes were used, each containing from 0.1 cc. to 5 cc. of raw milk and made up to 10 cc. with boiled milk. The experimental tubes contained 5 cc. of the sample under investigation, and 5 cc. of boiled milk. To each of the tubes, both experimental and scale, 0.3 cc. of 1 % hydrogen peroxide and 0.25 cc. of 2 % *p*-phenylenediamine were added. Half an hour was allowed to elapse between the addition of the reagent and the comparison of the samples with the scale. After that period no perceptible change could be discerned to take place in the shades of the tubes during the time of the estimation. A fresh scale was made up for each experiment and the raw milk employed invariably was of the same sample as the milk used for investigation. New solutions of hydrogen peroxide and *p*-phenylenediamine were also made up every time, for the former is very unstable and the latter, like guaiacum tincture, after remaining for some time in solution, will react with milk without the addition of hydrogen peroxide. The experimental error of this method varies from 5 % when the peroxidase content is high to 2 % when the amount of peroxidase to be estimated is low.

The milk for investigation was heated in a beaker covered with a cork lid, through which passed a thermometer graduated in tenths of a degree and a stirrer, and which was provided with an opening for the removal of

the samples. The beaker was placed in an asbestos-jacketed water-bath, having a layer of liquid paraffin on the surface to diminish the evaporation, and regulated by a toluene regulator. Like the beaker, the bath contained a stirrer driven by a water-motor. In starting the experiment the milk in the beaker was quickly brought up to the desired temperature in a boiling water-bath, and then without loss of time placed in the regulated bath and the stirrers set going. As soon as the temperature in the beaker became constant the first sample was taken. The taking of the samples was then repeated after the desired intervals. In removing the samples warmed pipettes were used in order not to cool down the milk in the beaker. The test tubes containing the freshly withdrawn samples were immediately placed in cooled water so that the temperature of the milk was rapidly brought down. The entire procedure of removing the samples was carried out regularly in ten seconds.

DETERMINATION OF THE TEMPERATURE COEFFICIENT.

In order to determine the temperature coefficient of the rate of inactivation by heat of the peroxidase in milk, it was necessary to ascertain the constants for several temperatures from which the coefficients could be calculated. Preliminary experiments showed that heating of milk at 65° C. for four hours made hardly any perceptible impression on the peroxidase content. On the other hand, at 72° C. the inactivation of the enzyme proceeded at a rate much too fast for any reliable results to be recorded. Temperatures of 71°, 70° and 69° were found to be very convenient and were accordingly chosen for the determination of the constants. The following tables give the results obtained for these three temperatures.

TABLE I.

Time of heating in minutes	Temperature of milk	Percent. of peroxidase	Percent. of peroxidase calculated from $K=0.005066$
0	69.05° C.	63	77
20	68.95	61	61
40	68.9	49	48.3
60	68.9	37	38.2
80	68.9	31	30.3
101	68.9	25	24
120	68.85	19	19
140	68.85	17	15

$$K = \frac{1}{t} \log \frac{C_1}{C_2} = 0.005066 \text{ where } t = 100 \text{ mins., } C_1 = 61, C_2 = 19$$

TABLE II.

Time of heating in minutes	Temperature of milk	Percent. of peroxidase	Percent. of peroxidase calculated from $K=0.01333$
0	70.25° C.	61	55.7
10	70.1	41	41
20	70.1	30	30.1
50	70.07	12.5	12
60	70.05	9.5	8.8
70	70.05	6.5	6.5
80	70.05	5.5	4.8

$$K = \frac{1}{t} \log \frac{C_1}{C_2} = 0.01333 \text{ where } t = 60 \text{ mins., } C_1 = 41, C_2 = 6.5.$$

TABLE III.

Time of heating in minutes	Temperature of milk	Percent. of peroxidase	Percent. of peroxidase calculated from $K=0.02698$
0	71.15° C.	37	35.5
5	71.08	26	26
10	71.05	18	19.1
15	71	13	13.9
20	70.98	9.5	10.2
25	70.98	8	7.5
30	70.9	5.5	5.5
35	70.9	4.5	4

$$K = \frac{1}{t} \log \frac{C_1}{C_2} = 0.02698 \text{ where } t = 25 \text{ mins., } C_1 = 26, C_2 = 5.5.$$

The last column in these tables gives the percentage of peroxidase calculated from the constant (K) obtained from the equation of a unimolecular reaction. The first and last readings were not utilised for calculating the constant, as the possibility of error was more likely in these cases than in any other. In the first sample the temperature is usually not quite constant and the high content of the enzyme makes accurate estimation harder than in the other samples, while in the last sample the experimental error of the colorimetric method is too significant in comparison with the low peroxidase content to be determined. The second reading and the last but one were therefore employed. Considering that the method which was employed for the estimation of the enzyme cannot claim a very high degree of accuracy, the figures calculated from the constants agree well with the figures actually obtained by experiment and this shows clearly that the inactivation of the enzyme proceeds as a reaction of the first order. It is also to be observed that constants obtained by calculating from different data in the tables gave results which approximated to those given above. The error in

the constant is probably between 5-10 %. Fig. 1 gives a graphic representation of the rates of inactivation at the three temperatures.

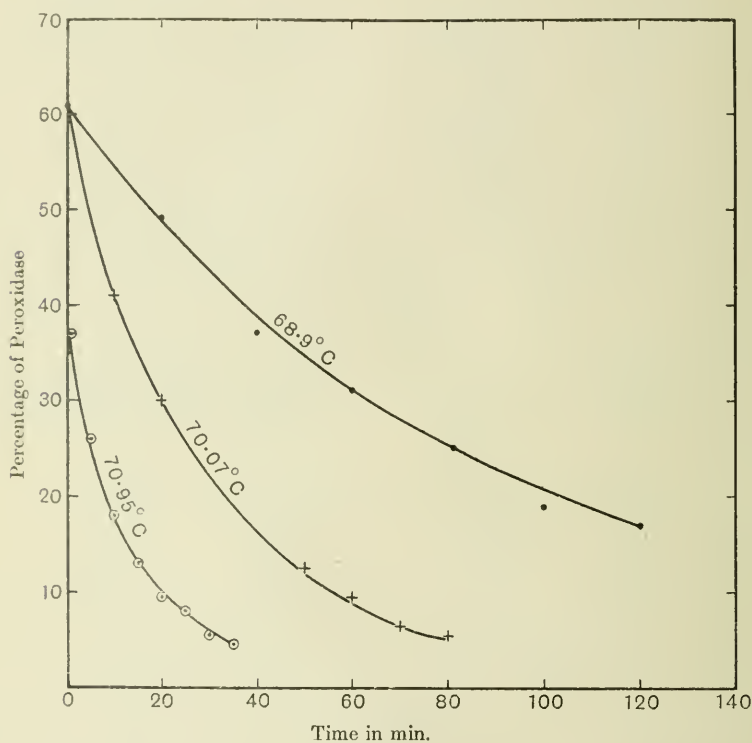


Fig. 1.

Calculating the temperature coefficient from the three constants, we obtain the following figures:

TABLE IV.

Temperature	Constant	Difference in temperature in degrees	Temperature coefficient for one degree
68.9° C.	0.005066	—	—
70.075	0.01333	1.175	2.278
70.95	0.02698	0.875	2.18
70.95	0.02698	2.05	2.236
Mean temperature coefficient	..	..	2.231

The mean temperature coefficient is therefore 2.23 per degree. This high value is of the same order as that found by Famulener and Madsen [1908] for the inactivation of tetanolysin and vibriolysin nasik by hot water. For the former the authors obtained the value of 2.4 and for the latter a value

a little under 2 for one degree. Chick and Martin [1910] also found the temperature coefficient for the coagulation of egg albumin by heat to be equal to 1.9 per degree.

The results of Tables I, II and III moreover show the impossibility of applying the peroxidase reaction as a direct test for pasteurisation. The term "pasteurisation" has a very broad application as to the temperature and time of heating the milk. It is not uncommon in some dairies in "pasteurising" to heat the milk up to 80° C. (176° F.), but according to the definition laid down by the Department of Health of the City of New York, pasteurisation consists in heating the milk at not less than

158° F. (70° C.)	for at least	3 mins.
155° F. (68.3° C.)	"	5 "
152° F. (66.7° C.)	"	10 "
148° F. (64.4° C.)	"	15 "
145° F. (62.8° C.)	"	18 "
140° F. (60° C.)	"	20 "

From the constant obtained above for 70° C. it can be calculated that 91.2 % of the original peroxidase would remain after heating the milk for 3 minutes at 158° F. (70° C.). Such an amount of peroxidase could be differentiated with difficulty by the colorimetric method from the full amount of 100 %, even if the original sample of raw milk could be used for the scale. In testing milk, however, for pasteurisation the original raw milk is never available and although different samples of raw milk differ apparently but little in intensity in the peroxidase reaction, the possibility of small variations is by no means excluded. This would make the differentiation still more uncertain. As regards the lower temperature, 20 minutes heating at 60° C. would make no perceptible impression, since, as we have seen, 4 hours at 65° C. makes hardly any difference to the peroxidase content of the milk. It is then evident that the peroxidase reaction cannot be applied at all as a test for pasteurisation. It may, however, serve as an indication whether "pasteurised" milk has been overheated.

TABLE V.

Temperature	Constant	Time in mins. necessary to bring peroxidase down to 10 %	Time in mins. necessary to bring peroxidase down to 50 %
70° C.	0.01333	75	22.5
71	0.02973	33	10.3
72	0.06634	15	4.6
73	0.14802	6.8	2
74	0.33022	3	under a minute
75	0.73673	1.3	" "
80	40.72004	under a minute	" "

Table V gives the periods of time for which the milk must be heated at different temperatures between 70° C. and 80° C. in order to bring down the peroxidase content to 10 % and 50 % respectively. The calculations are made on the assumption that the temperature coefficient remains the same as the temperature rises and the constants in the table are obtained on that assumption. It is clear from the figures that at temperatures beyond 71° C. very short periods of heating produce well marked impressions on the peroxidase content of milk.

INFLUENCE OF ACIDITY AND ALKALINITY ON THE RATE OF INACTIVATION OF PEROXIDASE IN MILK.

Experiments were instituted with the object of studying the effect of acidity and alkalinity on the rate of inactivation of peroxidase in milk. The H ion concentration of the milk was determined by the gas chain method. Unfortunately the determination of H ion concentration in a medium like milk is fraught with many technical difficulties, and the results obtained, though definitely showing the general effect of acidity and alkalinity, are not exact enough for publication in detail. The result arrived at is that a small increase in acidity retards and in alkalinity accelerates the rate of inactivation. Thus the addition of such a quantity of lactic acid as changed the P_H^+ of the milk from about 6.57 to 5.75 brought down the constant at 70° C. from 0.01333 to 0.01086, while the addition of potash which changed the P_H^+ to about 8.26 increased the rate of inactivation at the same temperature to such an extent that the peroxidase content of the milk came down to 2 % in the preliminary heating.

INFLUENCE OF SALTS ON THE RATE OF INACTIVATION OF PEROXIDASE IN MILK.

It was observed by Chick and Martin [1911] that the addition of sodium chloride and ammonium sulphate retarded the rate of coagulation of egg-albumin by heat. It was therefore desirable to ascertain the influence of salts on the rate of inactivation of peroxidase in milk by the same agency. From preliminary experiments it was observed that the addition of sodium chloride greatly retarded the rate of inactivation. In milk made up to the concentration of a N/1 NaCl solution the inactivation of the peroxidase at 70° C. was brought down to a rate too slow for convenient observation, and therefore N/10 and N/20 solutions were used in the actual experiments. The addition of salts alters the tone of the colouration produced by the

peroxidase reaction and salt was therefore added to the scale tubes in the same proportion as to the sample under investigation. Tables VI, VII and VIII illustrate the action of sodium chloride and Fig. 2 shows the same results graphically.

TABLE VI.

(N/10 NaCl.)

Time of heating in minutes	Temperature of milk	Percent. of peroxidase	Percent. of peroxidase calculated from $K=0.002063$
0	69.9° C.	72.5	72.5
30	69.9	59	62.9
90	69.9	48	47.4
120	69.9	42	41.1
150	69.9	36	35.6
180	69.9	32	30.9

TABLE VII.

(N/20 NaCl.)

Time of heating in minutes	Temperature of milk	Percent. of peroxidase	Percent. of peroxidase calculated from $K=0.003995$
0	70.2	72.5	72.5
31	70.15	50	54.5
60	70.1	41	41.8
90	70	31	31.7
120	70	25	24.1
150	70	20	18.25

TABLE VIII.

(Normal Milk, no salt added.)

Time of heating in minutes	Temperature of milk	Percent. of peroxidase	Percent. of peroxidase calculated from $K=0.01721$
0	69.9	62.5	62.5
5	70.0	50	51.3
10	70.0	40	42
25	70.1	24	23.2
40	70.1	14	12.8
55	70.2	8	7
70	70.2	4	3.9

Calculating $K = \frac{1}{t} \log \frac{C_1}{C_2}$ from different points on the curves in Fig. 2 we get the following mean coefficients for the corresponding concentrations:

N/10 NaCl, $K=0.002063$.N/20 NaCl, $K=0.003995$.Normal Milk, $K=0.01721$.

It is seen from the above figures that the concentration of N/20 salt reduces the rate to about one quarter and N/10 to about one eighth. The constant for the normal milk is rather higher than the one usually obtained for a similar temperature, but the same sample of milk was used for the experiments with the N/10 NaCl and N/20 NaCl and, consequently, these results would also be proportionately higher.

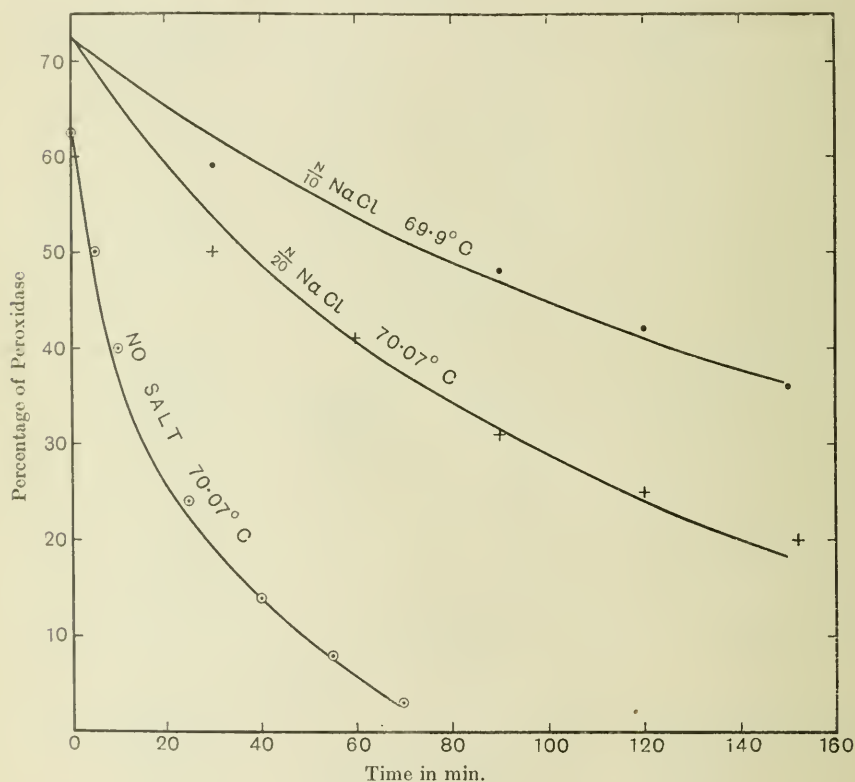


Fig. 2.

The next thing to ascertain was whether other salts had a retarding effect on the rate of inactivation, and if so whether that influence was specific or otherwise. The number of different salts used had to be restricted as some, even in such dilutions as N/20, bring down the caseinogen at 70° C. immediately, or after some time. In all the cases tried the addition of the salts, in concentration of N/20, had a marked retarding effect, although the extent of that influence varied in most cases. The influence of some of the salts is shown by the results in the following tables. Fig. 3 gives curves expressing these results as well as those in Tables VII and VIII.

TABLE IX.

(N/20 KCl.)

Time of heating in minutes	Temperature of milk	Percent. of peroxidase	Percent. of peroxidase calculated from $K=0.00526$
0	70.4° C.	65	65
15	70.5	56	54.2
30	70.5	51	45.2
45	70.5	38	37.7
60	70.5	29	31.4
75	70.5	24	26.2
90	70.5	20	21.8

TABLE X.

(N/20 Na₂SO₄.)

Time of heating in minutes	Temperature of milk	Percent. of peroxidase	Percent. of peroxidase calculated from $K=0.00546$
0	70.35° C.	77.5	75.5
15	70.32	62.5	62.5
30	70.35	53	51.8
45	70.3	42	42.9
60	70.3	33	35.5
75	70.3	28	29.4
90	70.3	20	24.3

TABLE XI.

(N/20 KNO₃.)

Time of heating in minutes	Temperature of milk	Percent. of peroxidase	Percent. of peroxidase calculated from $K=0.00981$
0	70.2° C.	72.5	82.8
15	70.4	59	59
30	70.35	40	42
45	70.4	28	30
60	70.4	20	21.35
75	70.35	16	15.2
90	70.4	10	10.8

Calculating the constants from the curves of Fig. 3 we get the following figures:

N/20 NaCl $K=0.00399$.
 N/20 KCl $K=0.00526$.
 N/20 Na₂SO₄ $K=0.00546$.
 N/20 KNO₃ $K=0.00981$.
 Normal milk $K=0.0225$.

The temperature for the normal milk and the N/20 NaCl was slightly lower than for the remainder. The constant of the normal milk has been calculated for the right temperature with the help of the temperature coefficient. The temperature coefficient for N/20 NaCl has not been deter-

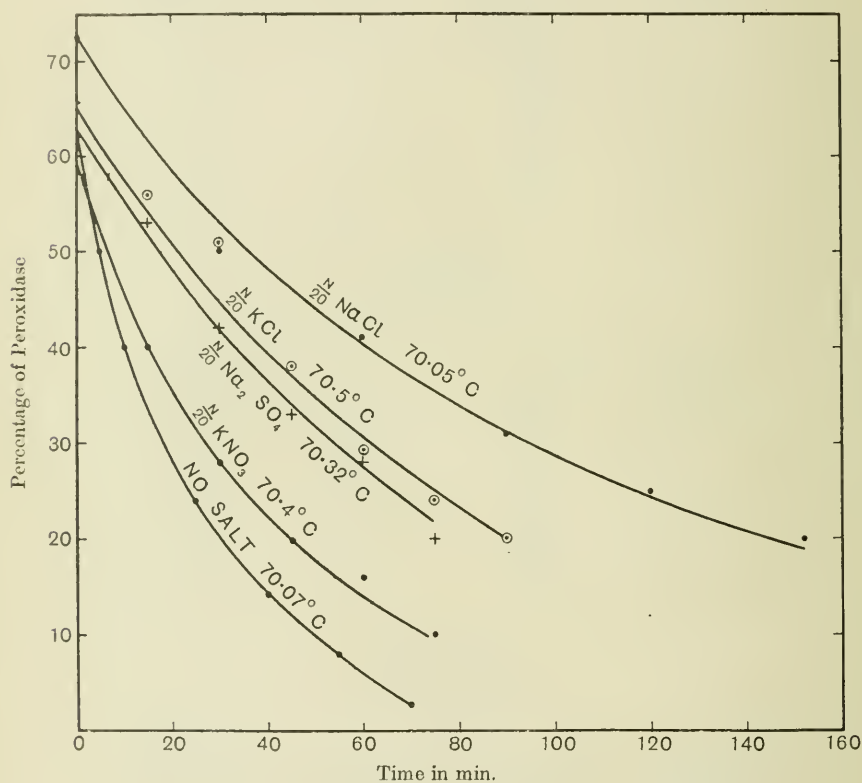


Fig. 3.

mined and consequently no correction could be made for that constant. The results seem to point to the fact that the influence is specific and is not dependent solely on the valency of the ions. KNO₃ and KCl contain monovalent kations and anions, but their constants differ considerably. On the other hand, KCl and Na₂SO₄ contain anions of different valency—the former monovalent and the latter divalent—yet their constants are practically the same.

THE INFLUENCE OF SALTS ON THE RATE OF INACTIVATION OF
PEROXIDASE IN WHEY.

The possibility that the salts act on the caseinogen, which then in its altered state influences the rate of inactivation of the peroxidase, was not excluded by the foregoing results and accordingly the effect of the addition of salts to whey was investigated. The whey was prepared by curdling the milk with rennet and filtering it through a cheese-cloth. The experiments were then carried out in the same way as with the milk. The inactivation of the peroxidase in the whey was also observed to proceed as a reaction of the first order but more rapidly than in the milk at the same temperature. The intensity of the peroxidase reaction in the whey was stronger than in the milk, and it was found convenient to dilute it (1 part whey with 3 parts boiled distilled water).

TABLE XII.

(Diluted Whey, no addition of Salt.)

Time of heating in minutes	Temperature of milk	Percent. of peroxidase	Percent. of peroxidase calculated from $K = 0.01485$
0	69.2° C.	72.5	72.5
15	69.35	47	43.4
30	69.3	28	26
45	69.3	14	15.6
60	69.3	9	9.3
75	69.3	5	5.5

TABLE XIII.

(Diluted Whey and N/20 NaCl).

Time of heating in minutes	Temperature of milk	Percent. of peroxidase	Percent. of peroxidase calculated from $K = 0.00344$
0	69.3° C.	72.5	72.5
15	69.3	62.5	64.3
30	69.3	55	57.2
45	69.3	50	50.8
60	69.3	42	45.1
75	69.3	40	40
90	69.3	38	35.5

Tables XII and XIII and Fig. 4 show the influence of N/20 NaCl on the diluted whey. As with milk, the retarding effect is well marked. N/20 KNO₃ was also found to produce a retarding effect.

Whatever the nature of the action of the salts may be, it is thus seen to be independent of the presence of the caseinogen and fat. Further investigation, which is now in progress, is needed in order to ascertain the nature of that action, and whether the enzyme is influenced directly or indirectly by the presence of the salts.

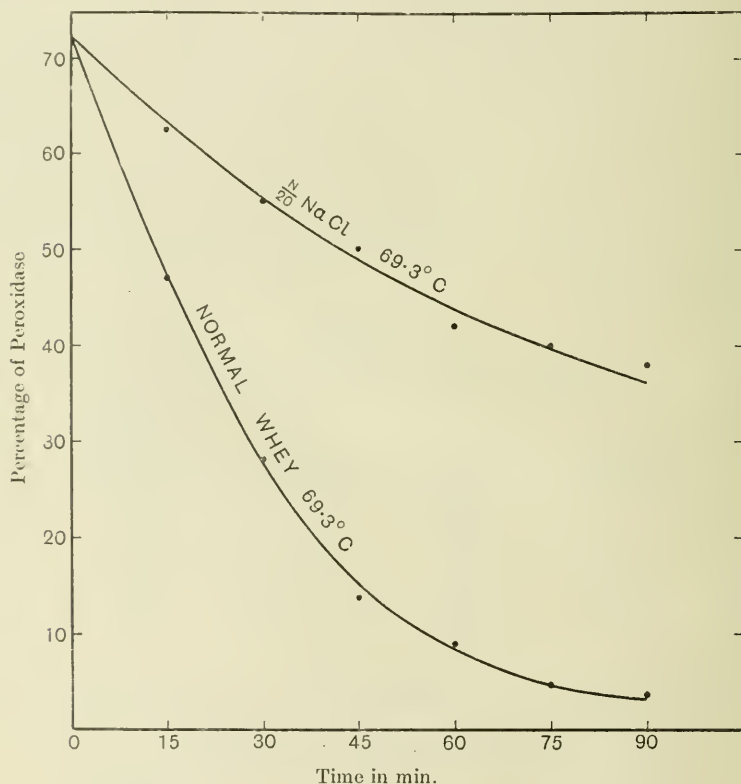


Fig. 4.

SUMMARY.

1. The temperature coefficient for the inactivation of peroxidase in milk by heat is found to be 2.23 per degree centigrade, a value of about the same order as the temperature coefficients for the inactivation by heat of principles like tetanolysin and vibriolysin nasik and for the rate of coagulation of egg-albumin.

2. The rate of inactivation of peroxidase in milk below 70° C. is so small that it does not afford the opportunity of utilising the peroxidase reaction

as a test for pasteurisation. The reaction may, however, indicate whether "pasteurised" milk has been overheated.

3. Small additions of acid retard and of alkali accelerate the rate of inactivation by heat of peroxidase in milk.

4. The presence of salts retards in a pronounced way the inactivation rate. This retardation varies with different salts and is independent of the valencies of their ions.

5. As in milk, the inactivation of peroxidase in whey proceeds as a reaction of the first order.

6. In whey also the presence of salts has a retarding influence on the rate of the enzyme.

In conclusion I wish to express my indebtedness to Professor Harden, F.R.S., for the valuable advice and assistance rendered during this investigation, and to Dr Onodera for helping me with some of the latter part of the experimental work.

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